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(54) USE OF COMMERCIAL LECITHIN AS SKIN PENETRATION ENHANCER

VERWENDUNG VON HANDELSÜBLICHEM LEZITHIN ALS HAUTPENETRATIONSFÖRDERER

UTILISATION DE LECITHINE COMMERCIALE EN TANT QU'AGENT AMELIORANT LA PENETRATION AU TRAVERS DE LA PEAU

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- **Patent Abstracts of Japan, vol. 8, no. 150, (C-233)(1587), 12 July 1984**
- **Chemical Abstracts, vol. 108, no. 12, 21 March 1988 (Columbus, Ohio, US), see p. 447, abstract no. 101343t**

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Description

Lecithin, as used in commerce, is the name given to a group of substances present in fats which are obtained mainly from oil seeds, for example, soybeans and rape seeds) and from egg yolks. Commercial lecithin is a natural emulsifying agent used in foodstuffs and pharmaceutical products, for example, in creams, balms, or ointments.

Lecithin has also been described in dispersions or suspensions in propellants. For example, bronchodilators can be administered by inhalation means in a propellant containing lecithin.

It has now been found that lecithin enhances the penetration of a drug through the skin and across other biological membranes, such as intestinal, buccal, rectal, and nasal. Thus an object of the present invention is a method of administering a drug transdermally or transmucosally by using lecithin in the pharmaceutical composition.

Accordingly, the present invention relates to a pharmaceutical composition adapted for transdermal or transmucosal administration comprising an active ingredient and an effective amount of lecithin.

Transdermal pharmaceutical compositions are used as a means for avoiding the uncertainties of oral administration and the inconvenience of administration by injection.

An active ingredient for purposes of the present invention is an effective amount of any therapeutically active drug.

Preferred drugs are those which find application in various therapeutic uses such as antihypertensives, analgesics, antitussives, antihistamines, bronchodilators and cognition activators. Therefore, the lecithin compositions are expected to be useful in drugs such as procaterol, dextromethorphan, oxymorphone and diphenhydramine.

The lecithin employed by the present invention is commercial lecithin or soy lecithin, for example, Lucas Meyer, Inc. commercial soy lecithins such as Epikuron™ 135F and Capcithin™ 50-R. The amount of lecithin used in the transdermal formulations may vary depending on the amount of active ingredient needed and the use of other excipients. A useful range of lecithin found to be effective for penetration enhancement is about 2 to about 40% by weight of the composition. Preferred is from about 5 to about 10%.

The present composition also may contain pharmaceutically acceptable excipients. These are, for example, tetraglycol (TG), alcohols, glycols, fatty acids, triacetin and silicon fluids.

The following examples in table form are illustrative of the present invention where the use of commercial soy lecithin was found to enhance penetration of various drugs through mouse skin.

TABLE

Formulation Number	Composition	Percent w/w	Drug Category	Flux (μg/cm²/h)	Flux Value in Absence of Lecithin (μg/cm²/h)
1.	Procaterol	2	Bronchodilator	93	Negligible
	EP 135 F	10			
	TG	88			
2.	CI-969*	2	Cognition Activator	110	5
	EP 135 F	10			
	TG	88			
3.	Dextromethorphan	2	Antitussive	214	10
	EP 135 F	10			
	TG	88			
4.	Dextromethorphan	2	Antitussive	119	10
	Cap 50-R	10			
	TG	88			
5.	Oxymorphone	4	Analgesic	25	Negligible
	EP 135 F	10			
	TG	86			
6.	Diphenhydramine	10	Antihistamine	570	~100
	EP 135 F	20			
	TG	70			
7.	Diphenhydramine	10	Antihistamine	1100	~100
	EP 135 F	10			
	TG	80			
8.	Diphenhydramine	10	Antihistamine	490	~100
	Cap 50-R	10			
	TG	80			

Code:
EP 135 F = Epikuron™ 135F (Lucas Meyer, Inc.)
TG = Tetraglycol
Cap 50-R = Capcithin™ 50R (Lucas Meyer, Inc.)

* CI-969 = Ethanone,1-(1,2,5,6-tetrahydro-1-methyl-3-pyridinyl)-0-acetyloxime.

Claims

1. Use of a commercial soy bean lecithin for the manufacture of pharmaceutical compositions enhancing penetration of a drug through the skin or other biological membrane, wherein the pharmaceutical compositions contain a therapeutically effective amount of an active ingredient, an effective amount of commercial soy bean lecithin and one or more pharmaceutically acceptable excipients with the proviso that the pharmaceutical composition is in a non-liposome form.

2. Use according to claim 1, wherein the active ingredient is an antihypertensive.

3. Use according to claim 1, wherein the active ingredient is an antihistamine.

4. Use according to claim 1, wherein the active ingredient is an analgesic.

5. Use according to claim 1, wherein the active ingredient is an antitussive.

6. Use according to claim 1, wherein the active ingredient is a cognition activator.

7. Use according to claim 1, wherein the active ingredient is a bronchodilator.

8. Use according to claim 1, wherein the active ingredient is procaterol.

9. Use according to claim 1, wherein the active ingredient is diphenhydramine.

10. Use according to claim 1, wherein the active ingredient is dextromethorphan.

11. Use according to claim 1, wherein the active ingredient is oxymorphone.

12. Use according to claim 1, wherein the effective amount of lecithin is 2 to 40 %.

13. Use according to claim 1, wherein the effective amount of lecithin is 5 to 10 %.

Patentansprüche

1. Verwendung von handelsüblichem Sojabohnenlecithin zur Herstellung von Arzneimittelzubereitungen erhöhter Arzneimitteldurchdringung durch die Haut oder eine sonstige biologische Membran, wobei die Arzneimittelzubereitungen eine therapeutisch wirksame Menge eines aktiven Bestandteils, eine wirksame Menge handelsüblichen Sojabohnenlecithins und einen oder mehrere pharmazeutisch akzeptablen (akzeptable) Streckmittel enthält unter dem Proviso, daß die Arzneimittelzubereitung in einer nicht liposomen Form vorliegt.

2. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem blutdrucksenkenden Mittel besteht.

3. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem Antihistamin besteht.

4. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem Analgetikum bzw. Schmerzmittel besteht.

5. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem Antitussivum bzw. Hustenmittel besteht.

6. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem Wahrnehmungsaktivator besteht.

7. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus einem Bronchodilatator besteht.

8. Verwendung nach Anspruch 1 wobei der aktive Bestandteil aus Procaterol besteht.

9. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus Diphenhydramin besteht.

10. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus Dextromethorphan besteht.

11. Verwendung nach Anspruch 1, wobei der aktive Bestandteil aus Oxymorphone besteht.

12. Verwendung nach Anspruch 1, wobei die wirksame Lecithinmenge 2 - 40% beträgt.

13. Verwendung nach Anspruch 1, wobei die wirksame Lecithinmenge 5 - 10% beträgt.

Revendications

1. L'utilisation de la lécithine de soja disponible dans le commerce pour la préparation de composition pharmaceutique améliorant la pénétration d'un médicament au travers de la peau ou au travers d'autres membranes biologi-

ques, caracterisée en ce que les compositions pharmaceutiques contiennent une quantité efficace du point de vue thérapeutique d'un principe actif, une quantité efficace de lécithine de soja disponible dans le commerce et un ou plusieurs excipients pharmaceutiquement acceptables sons la reserve, que les compositions pharmaceutiques ne contienent pas une formulation a base de liposome.

2. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est un anti-hypertenseur.

3. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est un anti-histaminique.

4. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est un analgésique.

5. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est antitussif.

6. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est un activateur de cognition.

7. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est un broncho-dilatateur.

8. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est le procatérol.

9. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est la diphenhydramine

10. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est le dextrométhorphan.

11. L'utilisation selon la revendication 1, caractérisée en ce que le principe actif est l'oxymorphone.

12. L'utilisation selon la revendication 1, caractérisée en ce que la quantité efficace de lécithine est comprise entre 2 et 40%.

13. L'utilisation selon la revendication 1, caractérisée en ce que la quantité efficace de lécithine est comprise entre 5 et 10%.

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(54) **Transnasal transport/immunisation with highly adaptable carriers**

Transnasaler Transport bzw. Impfung mit hochadaptierbaren Trägern

Transport/immunisation transnasale avec véhicules très adaptables

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Description

[0001] The invention deals with the transport of preferably large molecules across nasal mucosa by means of specially designed, highly adaptable carriers loaded with said molecules. One of the purposes of making such formulations is to achieve non-invasive systemic delivery of therapeutic polypeptides, proteins and other macromolecules; the other intent is to overcome circumstantially the blood-brain barrier by exploiting the nasal cavity to enter the body and then to get access to the brain. A third intent is to achieve successful protective or tolerogenic immunisation via nasal antigen or allergen administration.

[0002] Nasal delivery has been explored extensively over the last decades and was discussed repeatedly as an alternative to the systemic delivery of drugs, especially peptides and proteins, which normally must be injected. Nasal delivery also attracted interest owing to the fact that it avoids the hepatic first-pass effect, the problem of degradation in nasal cavity notwithstanding, which creates a pseudo-first-pass effect (Sarkar, 1992). The latter difficulty prompted chemical or recombinant structural peptide or protein modifications to improve the stability and minimise the enzymatic cleavage of macromolecules in the nose (Wearley, 1991).

[0003] Some earlier reviewers (Ilium, 1991; Wearley, 1991) expected that transnasal peptide delivery, supported by absorption enhancers, will provide a convenient, efficient means for the administration of protein and peptide therapeutics. More recent surveyors took less optimistic stance, however (Harris, 1993). Rapid metabolism and nonlinear pharmacokinetics of nasally delivered peptides (Wearley, 1991) are partly responsible for this. The other reasons are the anatomical and temporal barriers presented by the nasal mucosa (Sarkar, 1992), and especially the intolerable side effects of most, if not all, methods currently in use for nasal delivery. This holds also true for efforts to deliver compounds with the aim to generate a protective immune response transnasally, which would represent a more natural way of antigen presentation than encountered by conventional injection. The adverse side effects observed with transnasal immunisation experiments are mainly due to the presence of immunoadjuvants (such as Cholera toxin (CT) or its fragment B, heat labile protein from *E. coli,* keyhole limpet hemocyanin, or other substances with ADP-rybosilating activity, for example), and/or molecules with a permeation enhancing activity, in addition to the antigen in the formulation for nasal delivery. While the former may be toxic, the latter are irritating to the immunised subject. Selectivity of immune response, moreover, cannot be achieved with unspecific stimulatory agents. Moreover, there is substantial variability in the resulting immune response after nasal antigen administration, probably due to the difficulty of depositing the immunogen on the sites in the nasal cavity with the lowest transbarrier transport resistance.

[0004] Almeida et al., Journal of Drug Targeting 3 (1996), 455-467 discusses nasal delivery of vaccines and mainly describes the role of mucosal-associated lymphoid tissue in the process of immune response induction. The association of antigens with adjuvants and particulate carriers, such as microparticles, nanoparticles and liposomes is emphasized. On page 462, left-hand column, second full paragraph, lines 21 to 24, it is concluded that liposomes increase the humoral immunity through their ability to act as an antigen depot, supplying macrophages with free (released) or entrapped antigen. The nasal dosing with tetanus toxoid-containing liposomes, according to the authors, produced serum IgG titres similar to those obtained with a 10 times lower dose given by intramuscular route. The conclusion the person skilled in the art would draw from Almeida et al. is that antigens that are responsible for the induction of protective immunity against various pathogens should be attached to a suitable carrier with adjuvant (or sustained release) properties etc.

[0005] Almeida et al. neither anticipates the present invention nor renders it obvious.

[0006] The human nasal cavities with a total volume of 15 mL and a total surface area of 150 cm^2 - which amounts to more than 1 m^2 if one allows for the surface corrugations - are covered by mucus and a mucosa 2 mm to 4 mm thick. Most of the cavity surface is lined by a respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells. The resulting permeability barrier is related to that of the oral cavity, with which it communicates and which is covered by a keratinised barrier tissue. In either case, the cells in the barrier are tightly packed and often sealed with the specialised intercellular lipid arrangements. Moreover, in either case, the permeability barrier is lowered by the topical use of substances which compromise the quality and packing of such lipidic seals and/or which increase the probability for molecular partitioning into the barrier. Deviant from the situation encountered in the mouth, from the nose foreign substances are cleared into the nasopharynx by the cilia, with an average speed of 5 mm/min. An exception is the upper region of nasal cavity, which contains no cilia but is covered by a pseudo-stratified olfactory neuroepithelium. The nasal subepithelum contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation.

[0007] Nasal route of delivery has been relatively unsuccessful to date when used for high molecular weight substances. Use of permeation enhancers did not improve the situation sufficiently, largely due to the fact that such substances are generally poorly tolerated and of limited usefulness. The pharmacodynamics resulting from nasal drug delivery is also highly variable. Major reasons for this are the inconsistency in the site of deposition or in the delivery details, as well as changes in the mucous secretion and mucociliary clearance; the latter are compounded especially by the presence of al-

lergy, hay fever, and the common cold in treated subjects (Harris, 1993). Protein degradation in mucosa is important as well (Sarkar, 1992). Despite this, numerous studies were done with buserelin, vasopressin, cholecystokinin, calcitonin, growth hormone and related substances (e.g. GHRH), erythropoietin, G-CSF, interferon, insulin, gonadotropin hormone releasing hormones (GnRH), and vasopressin analogues, the results of which are reviewed briefly in the following.

Systemic delivery of large drugs through the nose

[0008] Hexarelin (GH analogue; MW ≈ 800). The GH response to the intranasal hexarelin administration (about 18 µg/kg) was not significantly higher than that induced by an injection of 1 µg GHRH/kg (Ghigo et al., 1996). On the other hand, the former kind of treatment did not significantly modify IGF-I but increased IGFBP-3 levels. Both IGF-I and IGFBP-3 levels were slightly but significantly increased by oral treatment with the drug as well (Ghigo et al., 1996).

[0009] Intranasal treatment with octreotide nasal powder, a somatostatin analogue (up to 2 mg TID, corresponding to a mean GH value below 5 µg/L during 8 daytime hours), was well tolerated, with only mild side effects and no significant changes in the nasal mucosa. An improvement of the clinical picture was registered in all patients after a few days of octreotide nasal powder administration. Positive correlation was found between GH and IGF-I, GH and IGFBP-3, IGF-I and IGFBP-3, insulin and IGFBP-3 and insulin and IGF-I during chronic (3-6 months) treatment (Invitti et al., 1996).

[0010] Cholecystokinin (MW ≈ 1050). The carboxy terminal octapeptide of cholecystokinin (CCK-8) has similar functions as native cholecystokinin (CCK), but lacks receptor selectivity and metabolic stability. Mediation of satiety via the A-receptor subtype can be used for management of obesity. This was also shown after intranasal administration of Hpa(SO3H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH2, the result of moving the N-methyl group from Phe to Asp, which inhibited feeding in beagle dogs (Pierson et al., 1997).

[0011] After intranasal (10 µg) and intravenous (0.25 µg and 2.5 µg) administration of an octapeptide derivative of cholecystokinin, the substance CCK-8 was shown to affect the auditory event related potential (AERP) in 20 healthy subjects. The effect was stronger in women than in men (Pietrowsky et al., 1996). Plasma CCK-8 concentrations after intranasal administration of 10 µg CCK-8 were comparable to those of 0.25 µg CCK-8 given i.v., but were substantially lower than those elicited by 2.5 µg CCK-8 (Pietrowsky et al., 1996).

[0012] Vasopressin (MW = 1054). Vasopressin DGAVP (2 mg) was administered intranasally and orally to healthy subjects for 1 week. Peak levels were always observed at 15 min. The mean absorption and elimination half-life (around 8 min and 35-38 min, respectively) were similar for the two tested routes of administration, but the latter only had 0.7 % relative bioavailability (Westenberg et al., 1994).

[0013] In a double-blind, crossover study, subjects received on three different occasions 20 IU of (arginine) vasopressin (AVP) intranasally (IN), or 1.5 IU of AVP and saline solution i.v.. Evoked potentials (ERPs) were recorded during the subject's performance on a auditory attention task. Plasma concentrations of vasopressin during task performance were enhanced after AVP, with the increase after i.v. administration of AVP exceeding 2000-fold that after AVP i.v.. Intranasal administration of AVP substantially increased the P3 component of the ERP in contrast to the injection (Pietrowsky et al., 1996).

[0014] Acute (2 mg) and chronic, 2 weeks treatment (1 mg/day) with nasal DGAVP revealed an improved short-term memory for abstract words in males but not in females, with no positive effect on learning concrete words. Chronic, but not acute, treatment with DGAVP reduced the reaction time for scanning of digits in a memory comparison task (Sternberg paradigm) in both sexes (Bruins et al., 1995). In a different human study, arginine-vasopressin (AVP: 3x10 IU) enhanced memory performance after nasal administration. The late positive complex (LPC) elicited by oddball stimuli was not affected whereas the structural encoding task revealed an effect of the drug. In both studies, AVP intake resulted in a marked change of the scalp distribution of the P3 component, which is a prominent part of the LPC. Vasopressin was thus concluded to influence the central nervous processing of the emotional content of stimuli (Naumann et al., 1991).

[0015] Subchronic treatment with vasopressin (40 IU/day) was shown to enhance nocturnal slow-wave sleep in 2 elderly subjects (Perras et al., 1996) However, the intranasal administration of vasopressin (DDAVP: 30 or 60 micrograms) had no general effect on pain perception in humans, but some other effects were observed (Pohl et al., 1996).

[0016] Buserelin (MW = 1239). Treatment of 40 women with endometriosis and 10 women with uterine leiomyoma by using GnRH agonist buserelin (200 µg, 3x daily, 6 months, intranasally) reduced AFS mean pelvic score from 24 to 7 and the size of the fibroids decreased by 69 % (Biberoglu et al., 1991).

[0017] Calcitonin (MW = 3432). Ichikawa et al. (1994) concluded that nasal (5, 10, 20 and 40 U/rat) and subcutaneous (5, 10 and 20 U/kg) administration of *Salmon calcitonin* on alternate days for 3 weeks, starting a week after ovarectomy, prevented the osteopenic changes, the invasive method being approximately 2-times more effective.

[0018] In a double-blind trial, the effect of intranasal administration of *Salmon calcitonin* on biochemical parameters of bone turnover in 32 patients immobilised for a prolapsed intervertebral disk was investigated (van der Wiel et al., 1993). Calcitonin in a dose of two times 200 IU/day inhibited by 40 % the increase in the fasting 2 h urinary hydroxyproline/creatinine ratio (OHPr/Cr)

and lowered by 80 % the increase in calcium / creatinine ratio (Ca/Cr). The decrease in serum 1,25-dihydroxyvitamin D after 10 days of immobilization was significantly less in the calcitonin-treated group than in the placebo group (14 versus 29%, respectively; $P < 0.05$). However, intranasal calcitonin, which was well tolerated, did not influence the pain scores as measured with a visual analog scale (van der Wiel et al., 1993).

[0019] Growth hormone (GH) releasing factor/s (MW = 5040). The current mode of growth hormone replacement therapy is daily subcutaneous (s.c.) injections given in the evening. This schedule is unable to mimic the endogenous pulsatile pattern of GH secretion, which might be of importance for the induction of growth and other GH actions (Laursen et al., 1996).

[0020] To simulate endogenous production of growth hormone the protein was administered on three occasions intranasally in doses of 0.05, 0.10 and 0.20 IU/kg, using didecanoyl-L-α -phosphatidylcholine as an enhancer (Laursen et al., 1996). On the other two occasions the patients received an s.c. injection (0. 10 IU/kg) and an i.v. injection (0.015 IU/kg) of GH, respectively. The nasal doses and the s.c. injection were given in random order in a crossover design. Intravenous administration produced a short-lived serum GH peak value of 128 µg/L. Peak levels were around 14 µg/L after s.c. injection (50 % bioavailability) and between 3 µg/L and 8µg/L, respectively, after the three nasal doses (bioavailability between 4 % and 9 %). Serum insulin-like growth factor I (IGF-I) levels increased significantly after s.c. administration only. However, the data revealed that a closer imitation of the physiological GH pulses was achieved via the nose. Despite this the authors of the study concluded that GH administration is of limited importance for the induction of a metabolic response to GH (Laursen et al., 1996).

[0021] GHRP-2 is one of the most potent members of the GHRP family, which exerts its biological activity after oral, intranasal and i.v. administration. For example, the children who had a robust response to the injected GH-releasing factors also received intranasal GHRP-2, with significant, but not quantitated, response over a dose range of 5-20 µg/kg per dose (Pihoker et al., 1995).

[0022] Insulin (MW = 5808). The problem of low bioavailability of insulin solutions given through the nasal mucosa was improved by using absorption enhancers or bioadhesive microspheres (Gizurarson & Bechgaard, 1991; Ilium & Davis, 1992). Bioavailability greater than 10 % was measured but to date no corresponding formulation has found its way into the late clinical trials. The chief reason for this appears to be the severe damage to nasal mucosa caused by the commonly used permeation enhancers.

[0023] For example, following the administration of powder formulations comprising insulin and the permeation enhancer sodium tauro-24,25-dihydrofusidate (STDHF), the hypoglycaemic response and the serum insulin levels in sheep increased with STDHF/insulin molar ratio in the range 0 to 16.8 (Lee et al., 1991). The reason for this is increased mucosal permeability as well as reduced insulin aggregate size. The bioavailability ranges from 2.9 % to 37.8 % for the powder, and was reported to be 15.7 % and 37.4 %, respectively for the drops or spray containing STDHF/insulin = 8.4/1 mixture, and roughly proportionally to the enhancer concentration (Lee et al., 1991). To achieve a high bioavailability major changes in nasal mucosa had to be tolerated, however.

[0024] In humans, the 200 U insulin/mL formulation containing a blend of enhancers (didecanoyl-phosphatidylcholine (2 w-%), glycerol (1.6 w-%), 0.4 w-% fractionated coconut oil) and 0.2 w-% cholesterol resulted in appr. 8 % bioavailability, the highest values having been measured for the high dose (2x3 sprays of 50 µL each), which also was most irritant (Drejer et al., 1991).

[0025] Cyclodextrins dissociate insulin hexamers into smaller aggregates, in dependence on structure and concentration. Hexamer dissociation was therefore speculated to be the reason for higher nasal absorption of the polypeptide (Shao et al., 1992). The relative effectiveness of various cyclodextrins for this purpose was reported to decrease from dimethyl-β-cyclodextrin (DM-β-CD) > α-cyclodextrin (α-CD) > β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) > γ-cyclodextrin (gamma-CD). A direct relationship between absorption promotion and nasal membrane protein and lipid release was invoked to explain such sequence (Shao et al., 1992).

[0026] It is less clear why cationic chitosan enhances the absorption of insulin across the nasal mucosa of rat and sheep in a concentration dependent fashion, with optimum concentrations higher than 0.2 % and 0.5 % in rats and sheep, respectively, but overall efficiency of this procedure is only around 10% (Ilium et al., 1994). Using didecanoyl-L-α-phosphatidylcholine as an enhancer results in 4 % to 9 % of nasal insulin bioavailability (Laursen et al., 1996).

[0027] G-CSF (MW = 19600). The relative bioavailability of rhG-CSF administered nasally in the rat was approximately 2%, compared to an s.c. injection, as evaluated from the immunologically active rhG-CSF concentration in rat plasma and the area under the curve (AUC) at t=8 h. Leukocyte stimulation counts suggested 5-10% availability at t=48 h. Relative bioavailability and pharmacological availability were increased 23 times and 3 times, respectively, by polyoxyethylene 9-lauryl ether (Laureth-9), but no increase in availability occurred with sodium glycocholate (Machida et al., 1993).

[0028] Absorption of dissolved recombinant human granulocyte colony-stimulating factors (rhG-CSF at pH 4)) through the nose of rabbits was investigated with dimethyl-β-cyclodextrin added or without such excipient, which acts as barrier permeation enhancer. The proteins were absorbed and the total leukocyte numbers in peripheral blood increased in either case, but excipients improved the absorption of rhG-CSF appreciably

(Watanabe et al., 1993). A subsequent pharmacokinetic and pharmacodynamic study (Watanabe et al., 1995) revealed that protein is absorbed through the nasal cavity from a solution, especially in the presence of alpha-cyclodextrin (α-CyD), which can act as carrier in the membrane. Good correlation was found between the logarithm of the area under the serum G-CSF concentration-time curve (AUC) and the area under the increased total blood leukocyte count-time curve (Watanabe et al., 1995).

[0029] Interferon (MW = 23000). Treatment of experimental rhinovirus colds in 38 adults by intranasal administration of recombinant interferon beta serine (MW = 18500) had no effect on illness rate or severity, but did decrease the frequency of virus shedding by the factor of 2 (on day 4) to 3 (on day 6). The course of middle-ear dysfunction associated with experimental colds was also positively affected by the drug (Sperber et al., 1992).

[0030] Erythropoietin (MW = 30400). The pharmacological availability of rh-EPO after intranasal administration without enhancers was compared to that of intravenous injections. The pharmacological activity was enhanced in low pH and hypotonic mannitol solution, which both compromise the barrier quality. This resulted in relative bioavailability of nasally applied drug between 7 % and 4 %, when estimated by different reticulocyte counting methods. (Shimoda et al., 1995).

[0031] Labelled dextrane (MW = 4100, 9000, 17500), applied nasally at the dose of 6.5 mg, was seen to pass mucosa in the presence of glycocholate (3 mg) and found in the blood in concentration range between 6 ng/mL and 21 ng/mL, which corresponds to app. 0.05 %, 0.02 %, and 0.01 % for the three molecular sizes, respectively (Maitani et al., 1989).

[0032] In summary, the combined teachings of the prior art demonstrated that the likelihood of large molecules to pass nasal mucosa decreases strongly with increasing molecular weight. To date, the size of molecules administered successfully through the nose is typically < 1300 Da, and always below 3500 Da. Significant transport is achieved only with supporting permeation promotors and is, in a certain concentration range at least, proportional to the enhancer concentration. Enhancer concentration in the percentile range can ensure up to 30 % drug (or label) bioavailability but more often values below 10 % and typically of a few percent are obtained. High transfer efficiency is accompanied with strong local tissue damage. This causes unpleasant acute side effects and may, first, abrogate the nasal permeability barrier and, upon repeated use, provoke extensive keratinisation of the epithelium that finally reduces transnasal transport efficiency.

[0033] The success of transnasal transport is believed to rely on the loosening of ciliated-goblet, goblet-goblet, or ciliated-ciliated cell contacts, which also opens passages for the motion of water (McMartin et al., 1987). Procedures or substances which support the process either osmotically (as in the case of polysaccharide addition), physico/chemically (as in the case of surfactant addition) or biologically (as in the case of molecules which affect the cell biochemistry, including many drugs, cell adhesion or trans- and epicellular transport), can therefore improve drug delivery across the nasal mucosa. Translocation through the cells is possible, but probably rare, except, maybe, in the cases of some viral infections or applications. Materials, such as polymers of polyelectrolytes, which prolong the retention time of and increase the proximity between the molecules to be transported and cellular membranes, are useful for the purpose as well. The limit to this latter effect is set by ciliary motion, which tends to clear mucosal surface approximately every 30 min and transports the superficial material into the throat, and thus towards the gastrointestinal tract. Transport mediated by certain particles was contended to rely on this effect.

Particle delivery through the nose

[0034] Inhaled fine particles (Kanto loam dust, fly ash, carbon black, diesel exhaust particles (DEP), and aluminium hydroxide (alum)) appear to act as adjuvants, and accelerate the production of IgE antibody against pollen in female BDF1 mice; however, the nature of the particles, their capacity to adsorb antigens, and/or their size seem to play only minor role in the process (Maejima et al., 1997).

[0035] Hollow spheres, according to Ting et al. (1992), are unsuitable for nasal delivery, owing to their rapid clearance and variable deposition pattern. Polyvinyl alcohol microparticles in the form of collapsed, solid spheres with the desired size for nasal deposition (10-200 μm) were therefore produced by spray-drying and spray-desolvation (Ting et al., 1992).

[0036] The above observation notwithstanding, several kinds of particulate suspensions were used in the nose, typically to elicit antibodies against the particle-associated antigens.

[0037] This includes so-called proteosomes comprising gp160 (Lowell et al., 1997) or influenza virus proteins. Another example are particles made from polymerised carbohydrates coated with a lipid (bi)layer.

[0038] It is important to realise, however, that in any nasal uptake study one should consider and allow for secondary redestribution. For example, the biodistribution of radioactivity from the purified major *Parietaria judaica* allergen after sublingual, oral, and intranasal administration in healthy human volunteers is similar. This is indicative of test material swallowing and absorption in the gastrointestinal tract (Bagnasco et al., 1997). In the intranasal case, transport to the pharynx by mucociliary clearance plays an important role as well, but a relevant fraction of the tracer is retained on the nasal mucosa for up to 48 hours after administration (Bagnasco et al., 1997).

Oral spill-over and the danger of false positive results

[0039] Proteins are absorbed in the gastrointestinal tract, albeit in small quantities. For example, ovalbumin (OVA) is absorbed in the stomach as well as from the GI tract into the blood and lymph circulation at levels of 0.007-0.008 % and 0.0007-0.002 % of applied dose; a higher dose in the latter case leads to relatively higher absorption (Tsume et al., 1996). Stomach absorption supplies nearly exclusively the blood, suggesting different mechanisms and/or routes of absorption between the stomach and the small intestine. OVA association with liposomes can improve the uptake about 2 to 3-fold, possibly owing to slower enzymatic degradation of OVA.

[0040] Often, the result of nasal and oral immunisation are very similar, suggesting that part of the effect of the former may be due to the spill over of the antigen into gastro-intestinal tract. Data obtained with human adenovirus type 5, used as a vector for heterologous DNA sequences, illustrate this (Flanagan et al., 1997).

Transnasal delivery into the central nervous tissue (CNS)

[0041] The access of substances to the brain is of paramount importance for the treatment of psychiatric and neurologic diseases. Transnasal route of delivery into the CNS was therefore tested for a few selected bioactive molecules.

[0042] To date, drug delivery into the CNS tissue by nasal administration has received little attention (Pesechnik & Price, 1996). Wheat-germ agglutinin coupled to horseradish peroxidase was demonstrated to be taken up by the cells of olfactory nerve, resulting in concentration in the olfactory bulb around 0.1 % of applied concentration; the underlying principle is probably receptor-mediated endocytosis of WGA and subsequent trans-synaptic, retrograde transfer towards the brain. A similar mechanism is also possible in the case of viral infections in the nose.

[0043] For example, an intranasal instillation of vesicular stomatitis virus (VSV), a negative-sense RNA virus, may result in a lethal infection of murine and rat brain (Huneycutt et al, 1994). Within 12 h following intranasal inoculation of VSV, this antigen can be detected in the olfactory nerve layer of the ipsilateral olfactory bulb. Within 3-4 days post-inoculation (p.i.), VSV had disseminated into the glomeruli of the olfactory bulb as well as the anterior olfactory nuclei, ipsilateral to the VSV instillation. Within the glomeruli, VSV antigen is more prevalent in the granule cells than in the mitral cells. Correspondingly, the lateral olfactory tract, where axons of mitral cells course, remain VSV negative throughout 7 days p.i.. By 7 days p.i., viral proteins are detected in several additional regions extending to the brainstem. The pattern of VSV immunoreactivity supports the picture of initial infection of the olfactory bulb glomeruli, with subsequent spreads via both ventricular surfaces and retrograde transport within axons of neuromodulatory transmitter systems enervating the olfactory bulb (Huneycutt et al, 1994).

[0044] Draghia et al. (1995) have demonstrated that it is possible to transfer the *Escherichia coli* lacZ gene in vivo into the central nervous system structures of rats after nasal instillation of replication-defective adenoviral vector AdRSV beta gal. Mitral cells from the olfactory bulb, neurons from the anterior olfactory nucleus, locus coeruleus and area postrema expressed beta-galactosidase for at least 12 days (Draghia et al., 1995). Parainfluenza type 1 vaccine virus also directly accesses the central nervous system by infecting olfactory neurons (Mori et al., 1996).

[0045] However, it would be highly desirable to have a convenient and reliable transnasal transport system for the compounds that are capable of and intended to generate a protective immune response without simultanously generating a variety of adverse side effects. Common types of non-invasive applications, including oral immunisation, often do not elicit the desired immune response. Many injectible vaccines also do not provide optimum antibody isotype pattern, mainly due to the unnatural route of antigen entry into the body. Transnasal immunisation remains problematic owing to the large size of typical immunogen which is subject to similar restrictions as the transport of pharmaceutically active compounds across the nasal mucosa.

[0046] In conclusion, although the prior art has tested various approaches to transnasal delivery it has hitherto failed to provide a convincing principle for convenient and well tolerated transfer of compounds, such as pharmaceutically active substances, immunogens/antigens or allergens, through the nasal barrier, in particular if said compounds are large. The solution to said technical problem, i.e. the provision of a suitable system, is provided by the embodiments characterised in the claims.

[0047] Accordingly, the present invention relates to use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the unsolubilised droplet,

whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains as a carrier for the preparation of a pharmaceutical, preferably a vaccine composition for transnasal administration.

[0048] Pharmaceutically active compounds, antigens or allergens do not cross the nasal mucosa in a practically meaningful quantity on their own without causing inacceptable side effects.

[0049] As regards the above recited values of up to 99%, it is to be noted that values below 50 % of the former relative concentration are particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

[0050] Formulations including the above-referenced penetrants are described in detail in DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, and DE 44 47 287, which are incorproated herewith by reference. Relevant information useful for penetrant manufacturing and loading with various macromolecular actives, which are too big to permeate through the barrier, is given in patent application PCT/EP98/06750, also incorporated herewith by reference.

[0051] More general information on lipid suspensions can be found in the handbook dealing with 'Liposomes' (Gregoriadis, G., ed., CRC Press, Boca Raton, FI., Vols 1-3, 1987), in the book Liposomes as drug carriers' (Gregoriadis, G., ed., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids, which can be used conveniently to prepare bio-compatible immunopenetrants, are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

[0052] The manufacturing temperature for said penetrants is normally chosen in the 0 °C to 95 °C range. Preferably, one works in the temperature range 10-70 °C, most frequently at temperatures between 15 °C and 45 °C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in the composition or physical state. These temperatures can be determined by the person skilled in the art using his common general knowledge and the teachings of the various documents cited in this specification. (For reference: the skin temperature is normally around 32 °C.) Other temperature ranges are possible, most notably for the systems containing freezable or non-volatile components, cryo- or heat-stabilised formulations, etc..

[0053] If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in the cold (e.g. at 4 °C), with or without associated active agents. It is also possible, and sometimes sensible, to manufacture and store the preparation under an inert atmosphere, e.g. under nitrogen. The shelf-life of carrier formulation, moreover, can be extended by using substances with a small number of double bonds, that is, by a low degree of unsaturation, by choosing peroxide-arm ingredients, by including antioxidants, chelators, and other stabilising agents, or by preparing the agent loaded penetrants *ad hoc* or *in situ,* e.g. from a freeze dried or dry mixture.

[0054] The term "two forms of a substance" in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc..

[0055] "Non-invasive administration" or "non-invasive delivery" in this specification denotes application on or transport through the nasal mucosa.

[0056] "Nasal administration", in the context of this document, refers to applications of test material, whether by direct intranasal intubation, spontaneous sniff of a drop of the test fluid, or an inhalation of the sprayed test-fluid into the nose, independent of precise site of impact or deposition.

[0057] The term "penetration" in this application describes non-diffusive motion of large entities across a barrier. This process is believed to involve penetrant adaptation to the otherwise confining pores in the barrier, perhaps in association with a transient, selective, and reversible decrease in the barrier resistance.

[0058] The term "permeation" refers to a diffusion across the semipermeable barrier and is typically driven by the permeant concentration gradient across the barrier.

[0059] A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

[0060] A permeant, on the other hand, is a molecule diffusing, or at least capable of diffusion, across the semi-permeable barrier.

[0061] The above-referenced penetrant is typically an ultra-adaptable entitiy comprising several components. Said penetrant, in the widest sense of the word, is a supra-macromolecular body that can pass spontaneously through the permeability barrier with pores much smaller than the penetrant diameter, and thus transport material from the application to the destination site on either side of the barrier. In order to meet this goal, the penetrant must adjust its properties, most notably its deformability, to the shape and size of the pores in a barrier. This typically occurs under the influence of a strong driving force or a pressure acting on all molecules in the penetrant. Gradients which do not depend on the penetrant concentration, such as hydration or external electric potential difference across the barrier, were shown to serve this purpose.

[0062] Lipid aggregates in (quasi)metastable state, and of the nature described above in connection with the invention, most often behave as highly adaptable penetrants, especially when they have the form of a tiny droplet surrounded by one or a few membranes (bilayers) (Cevc et al., 1997; Cevc et al., 1998). Owing to membrane metastability, unusually high local bilayer curvature can develop at the sites of transient, local membrane destabilisation without compromising the overall aggregate integrity. From the composition point of view, such ultra-adaptable and self-regulating vesicles typically consist of a suitably chosen lipid mixture. In order to change conventional lipid vesicles, liposomes, into the optimised vesicles (Transfersomes) one can add, for example, suitable edge-activators into the aggregate membrane (Cevc et al., 1998). Alternatively, molecules which change the system deformability after complexation with or binding to the basic aggregate ingredient can be used. Often, but not necessarily, the activators belong to the class of surfactants below the saturation or solubilization concentration, which in the latter case gives rise to mixed micelles formation. This is important as solubilised lipids, in the form of mixed lipid micelles, can cross the pores sufficiently wider than the micelle diameter but are incapable of enforcing channel opening in the biological tissues, which can be widened and trespassed by the mixed lipid vesicles, however. The postulated reason for this — to which the applicant does not wish to be bound - is the much greater aggregation number of the latter kind of aggregate which translates into the greater sensitivity to external, transport-driving gradients, such as the water activity gradient, and which is then capable of paying the energetic price for the pore or channel opening in the barrier.

[0063] The present invention is, in view of the prior art, particularly surprising since ultradeformable lipid vesicles would seem unsuitable for the purpose of transnasal delivery taken that they were reported to date to cross barriers, such as skin, only under non-occlusive conditions, that is, in the presence of a strong trans-barrier water concentration gradient (Cevc et al. 1995; Paul and Cevc, 1995), which is believed not to exist in the strongly hydrated nasal mucosa.

[0064] It was unexpectedly found that macromolecules in association with highly adaptable penetrants, typically in the form of mixed lipid vesicles, are transported across nasal mucosa despite the high water content in this mucosa and in the exhaled air saturated with humidity. Concluding from the fact that several successfully tested formulations of such carriers caused no irritation in the nose it is inferred that the aforementioned transport does not rely on damaging the barrier, such damage being the reason for more conventional transport of macromolecules from a solution across the nasal mucosa. Rather than this, it is reasoned (wherein the applicant does not wish to be bound by theory) that said transport relies on the carrier penetration through the barrier, which should not occur in a very humid surrounding.

[0065] It is furthermore taught in accordance with the invention that increasing the concentration of the surface active molecules, which can act as permeation enhancers, decreases the efficiency of corresponding protein transport across the nasal mucosa, at least when the solubilisation point of the carriers has been reached. This finding is unexpected in view of the fact that the art teaches that the bioavailability of nasally administered macromolecules typically gets higher with increasing permeation enhancer concentration.

[0066] A third unexpected finding is that carrier-mediated delivery of macromolecules across the nasal mucosa can mediate a relatively efficient transport of large molecules into the central nervous system (CNS). The influx is seen relatively soon after the drug administration into the nasal cavity when the large molecules are associated with the carriers. This could be due to the transport of carrier-associated drugs across the nasal mucosa and subsequent uptake of drug-laden carriers into the olfactory nerve, through which the drug could be carried towards and into the CNS by the retrograde transport; such transport has already been postulated and was tested with individual molecules (Pasechnik-V; Price-J. Exp. Opin. Invest. Drugs; 5: 1255-1276); the approach was not used, to the best of the applicant's knowledge, in combination with particulates to date. An alternative explanation would involve the carrier-mediated macromolecular delivery into the peri-nasal lymphatic system, which has been reported to communicate with the central nervous system (Kida-S; Pantazis-A; Weller-RO. Neuropathol. Appl. Neurobiol. 1993; 19: 480-448).

[0067] A fourth surprising result achieved in accordance with the present invention is that the referenced penetrants allow a successful and preferably protective transnasal immunisation with large immunogens. The use of highly adaptable antigen- or immunogen-carriers for the purposes of immunotherapy is expected to or has been shown in accordance with the present invention to provide all the benefits of more conventional nasal vaccinations in addition to the safety and robustness of ad-

ministration. Improved safety would reflect the choice of the non-toxic and non-irritating carrier ingredients. Better reproducibility could result from the greater ability of the specially designed carriers, compared to that of the antigens or immunoadjuvants used alone to overcome the nose barrier. Taken the expectation that different carrier populations loaded with the individual antigens could be combined into a final multi-valent vaccine formulation the capability of invented technology to meet the trend in immunotherapy is given.

[0068] It stands to reason that non-toxic and "gentle" formulations containing merely bio-compatible or natural, body-like ingredients, which protect the body faster and/or better than the corresponding antigen injections, would be preferred to the latter and would have a substantial commercial value.

[0069] In accordance with the present invention it is recommended to choose the penetrant characteristics, especially the deformability, concentration, or composition of the mixed lipid aggregates, so as to control the rate or the efficiency of penetrant-mediated transport.

[0070] In the process of optimisation of the formulation and/or administration it may be convenient to determine the flux of drug or agent loaded penetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which act across the barrier, and then to describe the data by a convenient characteristic curve which, in turn, is employed to optimise the formulation or application further.

[0071] The pharmaceutically acceptable form of the agent may be given in a variety of final formulations, optionally, and depending on the purpose of the administration, in combination with diverse secondary agents. Such agents will be explained in more detail later in the text and may be, for example, bacterial compounds or other immunomodulations.

[0072] The carrier is combined with the pharmaceutically active ingredient prior to the administration, e.g. when formulating said pharmaceutical composition. As regards the further explanations, description of advantages etc., of this and the following embodiments, reference is made to the respective description in connection with the first embodiment described herein above. It is further to be understood in accordance with the present invention that more than one type of antigen, allergen or pharmaceutically active ingredient or combinations thereof may be formulated into said pharmaceutical composition.

[0073] Additionally, the present invention relates to the use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the unsolubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains in combination with a pharmaceutically active ingredient or an allergen or an antigen for the preparation of a transnasally administerable pharmaceutical composition for the treatment of infective diseases, endocrine disorders, preferably hypopituitarism, diabetes, hyperthyroidism, thyroiditis, most preferably Hashimoto's thyroiditis, subacute thyroiditis; adrenal disorders, preferably Addison's disease, secondary adrenal insufficiency, Cushing's syndrome; gastrointestinal disorders, preferably Crohn's disease, colitis; hemorrhagic diseases, preferably hemophilia, leukopenia, hypereosinophilic syndrome; musculoskeletal and connective tissue disorders, preferably rheumatoid arthritis, Sjögren's syndrome, Bechet's syndrome, lupus, scleroderma, polymyositis/dermatomyositis, polymyalgia rheumatica and temporal arthritis, polyarteriosis nodosa, Wegener's granulomatosis, mixed connective tissue disorder, ankylosing spondylitis, psoriatic arthritis, osteoarthritis, Paget's disease, sciatica, bursitis, tendonitis or tenosynovitis, epicondylitis, fibromyalgia, eosinophilic faciitis; neurological disorders, preferably pain, singultus, vertigo, seizure disorders, sleep disorders, transient ischemic attacks, spinal cord injury, demyelinating diseases, nerve root disorders, myasthenia gravis; psychiatric disorders, preferably drug dependence, neuroses, mood disorders, schizophrenic disorders, delusional disorders; for oncological purposes and/or for treatment in the field of gynecology, preferably for the treatment of dysmenorrhea, menopause, chronic anovulation, premature ovarian failure, endometriosis, infertility; and/or for treatment in the field of immunology, preferably transplant rejection, hyposensitation, allergen immunotherapy or prophylactic vaccination.

[0074] The term "allergen" is used in this invention to describe materials of endogenous or xenogenic, e.g. animal or plant, origin which result in an undesired immune response of the body exposed to such an allergen, often resulting in an acute hypersensitivity reaction. Allergising microbes or parts thereof (e.g. of mite), parts of plants (e.g. pollen) or animal (e.g. hair and skin debris), but also man made and inorganic substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed

to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the skin or not.

[0075] An "antigen" is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system. A macromolecular antigen is defined as an antigen that is known to or believed to cross spontaneously the nasal barrier only in quantity too small for the desired practical purpose. Thus, macromolecules are molecules that, on their own, do not cross the nasal mucosa in practically useful quantity without causing inacceptable side effects.

[0076] In a preferred embodiment of the use of the present invention the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an antiallergicum, antiarrhythmicum, antiatheroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, antidrepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum or antihypertonicum, an antihypotonicum, anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardiotonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flew agent, a haemostaticum, hypnoticum, an immunoglobulin or an fragment thereof or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly)peptide (derivative), an opiate, an opthalmicum, (para)-sympaticomimeticum or (para)sympathicolyticum, a protein (derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an inhibitor (antagonist) or a promotor (agonist) of the activity of any of above mentioned agents, or any combination of said active substances. It is preferred that said active ingredient does not itself cross the nasal mucosa in practically meaningful quantity without inacceptable side effects.

[0077] In another preferred embodiment of the use of the present invention the antigen is derived from a pathogen.

[0078] In the context of this invention, the term "pathogen" refers to an entity which through its presence in or on the body leads to or promotes a pathological state which, in principle, is amenable to or could profit from a preventive, curative or adjuvant immunotherapy.

[0079] In a most preferred embodiment of the use of the invention said pathogen belongs to the class of extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria*, gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas,* Diptheria, *Bordetella Pertussis*, and gram-positive bacteria (e.g. *Bacillus pestis, BCG),* particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis)* and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases; eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections, also belong in this group.

[0080] It is most preferred that the antigen, preferably the pathogen, is used in a purified, or even better in a pure form.

[0081] Pathogens causing major infective diseases such as hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases are particularly preferred as are eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections.

[0082] In another preferred embodiment of the use of the invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

[0083] In further preferred embodiment of the use of the present invention the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., or is a part of implantation material.

[0084] In an additional preferred embodiment of the use of the present invention said pharmaceutical composition comprises a compound which releases or induces cytokine or anti-cytokine activity or exerts such an activity itself.

[0085] The term "cytokine", as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1α and IL-1β, tumour necrosis factor (TNF), transforming growth factor (TGF-β and -α), Type I and II interferons (IFN-α1, IFN-α2, (IFN-ω), IFN-β, IFN-γ), migration inhibitory factor, MIF, c-kit ligand, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

[0086] Cytokines that mediate natural immunity particularly well include type I interferons (IFN-α and IFN-β), tumour necrosis factor (TNF), interleukin-1 (IL-1 α and IL-1β), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. Antiproliferative (e.g. with IFN-s), pro-inflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other, may be generated by transnasal administration of the pharmaceutical composition described in accordance with the present invention. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, consequently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic or prophylactic action.

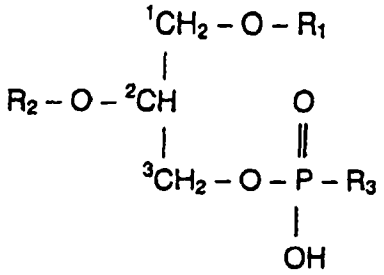
[0087] Cytokines that mediate the immune-mediated inflammation which heavily relies on the cell-mediated response are interferon-gamma (IFN-γ), lymphotoxin (TNF-β), interleukin-10 (IL-10), interleukin-5 (IL-5), interleukin-12 (IL-12) and, probably, migration inhibition factor. Leukocyte growth and differentiation are most affected by interleukin-3 (IL-3), c-kit ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage or granulocyte colony stimulating factor (M-CSF or G-CSF) and interleukin-7 (IL-7).

[0088] It is preferred to select the compound displaying cytokine activity amongst IL-4, IL-2, TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, amongst which IFN-alpha or IFN-β are most preferred, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

[0089] In another preferred embodiment said compound with anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative, or an analogue thereof.

[0090] In another preferred embodiment of the use of the present invention, the compound displaying or inducing cytokine or anti-cytokine activity and the pharmaceutically active ingredient or antigen or allergen are associated with the penetrant, e.g. in the form of a complex, hetero-aggregate, via encapsulation etc..

[0091] In an additional preferred embodiment of the use of the present invention the less soluble self-aggregating molecule is a lipid, preferably a polar lipid, and the more soluble component is a surfactant or some more soluble form of the polar/basic lipid. The former ingredient, typically, stems from a biological source or is a corresponding synthetic lipid or any of its modifications. Such lipid often belongs to the class of phospholipids with the chemical formula



where R_1 and R_2 is an aliphatic chain, typically a C_{10-20}-acyl, or -alkyl or a partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, or stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4}-alkyl, C_{1-5}-alkyl substituted with carboxy, C_{2-5}-alkyl substituted with hydroxy, C_{2-5}-alkyl substituted with carboxy and hydroxy, or C_{2-5}-alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phospha-

tidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebrosides, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyllipids, or belong to the backbone as in sphingolipids.

[0092] The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyl-dimethyl-aminoxide, esp. a dodecyl- dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N-dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl, -myristoyl, -cetylstearyl, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate, -linoleate, -linolenate-, - palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkyl-sulphate (salt), e.g. in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g. lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecyl-phosphatidylcholine, or else is a surface-active polypeptide. It is important to realise, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionisation or salt forms of the polar lipids may differ widely in their properties. It therefore stands to reason that two different physicochemical states of the same (polar) lipid mixed together in a membrane may produce a highly deformable carrier satisfying the conditions of this invention.

[0093] In an additional preferred embodiment of the use of the present invention, the more soluble component is an agent to be transported across the barrier, said agent having a tendency to form common large structures with the less soluble component(s) of the penetrant, typically in the form of a physical or a chemical complex.

[0094] In a further preferred use of the invention, the more soluble component tends to solubilise the penetrating droplet and is present in concentration not exceeding 99 mol% of the concentration required to disintegrate the droplet or, alternatively, not exceeding 99 mol% of the saturating concentration in the unsolubilised droplet, whichever is higher, values below 50 % of the former relative concentration being particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

[0095] In a different preferred embodiment of the use of the invention, the less soluble penetrant component is a lipid, preferably a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

[0096] In another preferred embodiment of the use of the present invention, the average penetrant diameter is between 25 nm and 500 nm, preferably between 30 nm and 250 nm, even more preferably between 35 nm and 200 nm and particularly preferably between 40 nm and 150 nm.

[0097] In a different preferred embodiment of the use of the present invention the penetrant concentration in the formulation for the use in the human or animal nose is 0.001 weight-% (w-%) to 20 w-% of total dry mass in the formulation, in particular between 0.01 w-% and 15 w-%, more preferably between 0.1 w-% and 12.5 w-% and most preferred between 0.5 w-% and 10 w-%.

[0098] In a further preferred embodiment of the use of the present invention the supporting medium, e.g. a buffer, is selected to be a biocompatible solution with an osmotic activity similar to that of a monovalent electrolyte with a concentration range between 1 mM and 500 mM, more preferably between 10 mM and 400 mM, even more preferably between 50 mM and 300 mM, and most preferably between 100 mM and 200 mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier. The term "practically sufficient penetrant stability" means that the penetrant stability meets the reasonable product quality criteria. The term "practically sufficient transport rate" means that enough drug is transported through the barrier without using unrea-

sonably large application volume or time. Said sufficient penetrant stability combined with sufficient transport rate across the barrier can be determined by the person skilled in the art without undue experimentation.

[0099] In another preferred embodiment of the use of the present invention, the relative drug or agent concentration is between 0.001 w-% and 40 w-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.

[0100] In one further preferred embodiment of the use of the present invention the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.

[0101] In another preferred embodiment of the use of the present invention additives are included in said composition to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc..

[0102] In a different preferred embodiment of the use of the present invention the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

[0103] In another preferred embodiment of the use of the present invention the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

[0104] The efficiency of administration and the biological effects of the agent or drug chosen, consequently, can be controlled by using different application volumes. Various metered delivery devices can be used for the purpose.

[0105] Accordingly, in an additional preferred embodiment of the use of the present invention said formulation is administered using a metered delivery device.

[0106] In one further preferred embodiment of the use of the present invention different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.

[0107] In a different preferred embodiment of the use of the present invention the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation is administrated in the nose. This embodiment is expected to improve the formulation stability, loading efficiency, the release kinetics, ease of use, compliance, etc..

[0108] In another preferred embodiment of the use of the present invention the, delivery device is loaded at the treatment site.

[0109] In a further preferred embodiment of the use of the present invention the delivery device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.

[0110] In one further preferred embodiment of the use of the present invention the pharmaceutically active ingredient is for administration to the nervous system.

[0111] The term "administration" in connection with this embodiment means that the pharmaceutical composition is transnasally applied, but the target site of the active ingredient is the nervous system, preferably the CNS and most preferably the brain. The possibility to use nasal application of the highly adaptable, drug-loaded penetrants in the nose to mediate a practically useful transfer of the drug across the barrier can thus be exploited to transport a meaningful amount of the drug, and to create a significant concentration of such drug, in the central nervous system or some other adjacent tissue, such as the eye.

[0112] In another preferred embodiment of the invention, the pharmaceutical composition is a vaccine.

[0113] Said vaccine can be used for therapeutic or prophylactic vaccination.

[0114] The term "(therapeutic) vaccination" in the context of this invention describes any kind of therapeutic immunisation, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

[0115] Immunisation denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

[0116] An "antibody" or an "immunoglobulin" denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their "derivatives" include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives Fragments include e.g. single chain fragments, Fc-, Fab- $F(ab')_2$- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

[0117] The term "immunogen" denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing

an immune response.

[0118] "Immuno-tolerance" denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

[0119] Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

[0120] Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

[0121] As has been indicated above, the successful immunisation with the vaccine through the nose is a significant step forward in the design of conveniently administrable vaccines that (a) are highly efficient over a wide range of immunogens of varying size and properties; (b) can be formulated together with certain cytokines, compounds that mediate cytokine activity or compounds that antagonize cytokine activity in order to specifically direct the corresponding immune response or to augment or suppress the same as may be desired; (c) do not depend on the perturbing injection by a needle; and (d) cause no irritating side effects. In addition, with the vaccine described in the invention, successful tolerogenisation may be achieved.

[0122] It has inter alia been found in accordance with the present invention that

- Tween-SPC micelles give protection significantly below that of the vaccine, suggesting that the small size of the carrier or the presence of surfactants alone does not suffice for a successful immunisation;
- orally administered immuno-carriers create lower specific antibody titers than the transnasally administered vaccine, as determined on the basis of absorbance measurements;
- the transnasal vaccine gives rise to higher specific IgG1 and IgG2 titers in the blood and to comparable IgG2a and IgM titers as compared to mixed micelles; all titers were, on top of this, higher than those generated by immunisation with SPC:cholesterol (1:1) liposomes.

[0123] When the transnasal vaccine is formulated together with a cytokine or an immunoadjuvant it is advantageous to use (blends of) bacterial extracts. Specific examples given in this application include monophosphoryl lipid A (MPL) and IL-12 or GM-CSF and IL-4. In principle, however, the vaccine may be formulated or applied together with any of the compounds mediating, inducing or displaying cytokine activity or with antagonists thereto that have been recited herein above.

[0124] It is preferred in accordance with the use of the invention that the vaccine further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.

[0125] Most preferably, said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chickenpox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus or rubella diseases.

[0126] It is additionally preferred that said vaccine further comprises an adjuvant.

[0127] The term "adjuvant" is used here to describe any substance which supports, augments, stimulates, activates, potentiates or modulates the desired immune response of either cellular or humoral type, specifically in the case of a prophylactic treatment by increasing the antigen specific immune response of any kind and in the case of therapeutic treatment often by supporting cell-mediated immunity. This can be achieved by the addition of suitable cytokines, their blends or suitable agonists and antagonists. The class of immunoadjuvants which indirectly contribute to the useful cytokine pool includes small chemical entities with an allergenic potential, such as certain allergenic (metal) ions, including but not limited to LiCl, $HgCl_2$, molibdenum, acids, bases and other irritating compounds, such as dicyclohexylmethane-4,4'-diisocyanate, ditrocarb (diethyldithiocarbamate), 2,4-dinitrochlorobenzene, isoprinosine, isophorone-diisocyanate, levamisole, (phenyl)oxazolone and alike, Swansonine, sizofran, phthalic anhydride, thymopentin, (fatty) alcohols, (fatty) amines, (fatty) ethers, ricin, or other suitable amphiphiles, many surfactants and chemical permeation enhancers, as well as derivatives or combinations thereof; furthermore, (low molecular weight) fragments of or derivatives from microbes, including lipopolysaccharides (such as LPS), cord-factor (trehalose-dimycolate) and other (poly)saccharides or (poly)peptides attached to membranes, used in sufficient quantity, acetylmuramyl-alanyli-soglutamin, and larger fragments of microbes, including bacterial exo- and endotoxins, or enterotoxins, such as cholera toxin and the heat labile toxin of *E. coli*, and their macromolecular fragments, such as A-chain derivatives, most, if not all, of which seem to posses ADP-ribosylating activity, the high potency immunoadjuvant LT holotoxin, etc., cell-wall skeleton, attenuated bacteria, such as BCG, etc. Less established examples include clostridial toxin, purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* outer membrane protein of group B *Neisseria meningitidis* (GBOMP), various other peptidoglycanes, etc.. Immunoadjuvants, in other words, include molecules that alter the uptake or presentation of antigens, activate or increase the proliferation of antigen specific lymphocytes, or interfere with the dominant control mechanism in the immune response, not just in the nose but also in the other immunocompetent tissues. (The mucosal adjuvant activity of ADP-ribosylating bacterial enterotoxins is a well established and known example for this.) On the other hand, mole-

cules which change the (relative) concentrations of cytokines or other immunoadjuvants, such as anti-immunoadjuvant antibodies or other agonists or antagonists of immunoadjuvants, also are immunoadjuvants in the sense of this invention. The same is true for molecules which affect lymphocyte homing, such as various selectins (LECAMS, e.g. various CD62-s), GlyCAM-1, MadCAM-1, VCAM-1, ICAM-1, hyaluronate, etc., and other chemokines, such as RANTES or MCP-1. Endogeneous group of immunoadjuvants furthermore comprises histamines, transfer factor, tuftsin, etc.. As many of the above mentioned immunoadjuvants do not have sufficient potency to ensure the desired effect after the non-invasive immunisation at too low, and sometimes too high, concentration or on their own, the functional definition of an adjuvant used in this work includes a fortiory sufficient and such modulation of cytokine concentration and distribution pattern in the body that results in mounting the desired therapeutic or prophylactic immune response. If required to gain clarity said modulation and its extent must be determined in a dedicated experiment, in which the specific cytokine levels are determined, using methods known to the person skilled in the field.

[0128] In a further preferred embodiment of the use of the invention, said adjuvant is lipopolysaccharide, such as lipid A or a derivative or modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly) saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a microorganism; an extract of a microorganism, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, an LT halotoxin, purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

[0129] It is most preferred in accordance with the use of the invention that the vaccine comprises a blend of MPL and IL-12 or GM-CSF and IL-4, when pure cytokines and their inducers are used.

[0130] In a different preferred embodiment of the use of the present invention the relative immunogen/antigen dose in said vaccine to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen that would have to be injected to achieve the desired biological effect. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

[0131] It is further preferred in accordance with the invention that in said vaccine the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

[0132] Different administration volumes may be selected to control the applied immunogen dose and the outcome of vaccination. Various metered devices can be used for the purpose.

[0133] A suspension of antigen-free penetrants may be loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before administering the resulting formulation in the nose.

[0134] At least one dose of vaccine may be administered.

[0135] This embodiment includes the repeated administration of the vaccine described in accordance with the use of the invention. Repeated administration includes repeated administration in the nose or one or more administrations in the nose in combination with conventional, e.g. parenteral administrations. In this connection, a kit may be advantageously made to comprise one or more containers, ampules or other kind of units comprising the vaccine.

[0136] The time interval between the subsequent vaccinations may be chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.

[0137] Repeated immunogen administration may be advocated to maximise the final effect of a therapeutic vaccination. It is proposed to use between 2 and 10, often between 2 and 7, more typically up to 5 and most preferred up to 3 immunisations, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined as described above or by some other suitable assessment method, or else to deem the effort as having failed. The time interval between subsequent vaccinations should preferably be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years, when a subject is being immunised for the first time. Rodents, such as mice and rabbits are advantageously immunised in 2 weeks interval, primates, e.g. monkeys and often humans, need a booster vaccination in 3-6 months interval.

[0138] The flux of penetrants that carry an immunogen through the various pores in a well-defined barrier may be determined as a function of the suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formu-

lation or application further.

[0139] The disclosure content of the documents cited throughout this specification are herewith incorporated by reference. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of Innovative Dermale Applikationen GmbH and bearing the title "Noninvasive vaccination through the skin".

[0140] The figures show:

Figure 1 illustrates the effect of nasal insulin administration by means of carriers in an insulin dependent diabetes mellitus patient, with the result of an i. v. injection of fast-acting insulin (Actrapid, Novo-Nordisk) shown in the inset for reference.

Figure 2 illustrates the glucodynamics in a healthy human volunteer following intranasal administration of insulin by means of Transfersomes. Inset gives the result of intravenous injection of similar formulation for reference purpose.

Figures 3a and 3b provide further examples measured with a healthy volunteer following intranasal administration of insulin formulations with inferior characteristics, believed to be due to too slow drug release from the carrier.

Figure 4 illustrates the capability of nasally administered cytokines, associated with Transfersomes, to affect the outcome of transnasal immunisation with tetanus toxoid.

Figure 5 illustrates the biodistribution of insulin-derived radioactivity in mice following nasal administration of the agent in transfersomes.

Figure 6 gives the corresponding results for interferon, as measured in mice.

Figures 7 illustrate the effect of changing aggregate size and/or deformability on TT specific immune response in mice treated with various mixed micelles, Transfersomes or liposomes loaded with TT. Panels a and b show antibody isotype patterns, and in panel c the total antibody titre, as expressed in absorbency change is given.

Figures 8 highlight the (small) effect of changing antigen dose (in the high dosage range) on transnasal immunisation of mice with TT by means of Transfersomes with or without lipid A derivative as an immunoadjuvant. In panel a, the results of total absorbance measurements are given, panel b shows the corresponding titration curves, and panel c gives the relevant antibody isotypes.

Figure 9 is organised in similar fashion to compare the outcome of intranasal, oral or subcutaneous TT administration using different antigen doses and purity.

For comparison, in figure 10, animal protection (survival) data are given for the experiments in which several doses and administration routes were compared.

Figure 11 presents a set of data on the effect of various cytokines, or their combination, on the murine immune response to TT administered into the nose by means of transfersomes, with s.c. data given for comparison. Panel a gives the absorbance and titre data and panel b contains the isotype distribution results.

Figure 12 deals with the effects of combining low and high molecular weight immuno-adjuvants (lipid a analogue and interleukin-12).

Figure 13 illustrates the effect of specific cytokine inducers of microbial origin. Cholera toxin (CT) is used for the purpose.

[0141] The examples illustrate the invention.

EXAMPLES

General experimental set-up and sample preparation

[0142] ***Conventional vesicles, liposomes***, comprised soy phosphatidylcholine (SPC; Nattermann Phospholipids, Rhone-Poulenc Rorer, Cologne, Germany). The suspension containing 10 w-% of the lipid in form of multilamellar vesicles was prepared by suspending the lipid in a buffer and then extruding the suspension through several polycarbonate membranes (with 800 nm, 400 nm, 200 nm and 100 nm pores, respectively) to narrow down the final vesicle size distribution. If required, as judged on the basis of optical inspection or the dynamic light scattering done after the latter steps, extrusions were repeated several (up to 5) times. In some cases, the vesicles were first extruded to a diameter of app. 50 nm and then frozen and thawed three times to enlarge the vesicles again, owing to intervesicle fusion. Subsequently, the formulation was passed through a micro-porous filter (100 nm; Poretics, CA), under pressure, to prepare the final suspension of oligo- or unilamellar vesicles.

[0143] ***Highly adaptable penetrants***, used in the described examples, typically had the form of ultradeformable vesicles (Transfersomes™) with one or a few bilayers. They comprised a mixture of phosphatidylcholine and (bio)surfactants (cholate or polysorbate (Tween 80)), and various biologically active ingredients, such as insulin, interferon, interleukin, or GC-SF.

[0144] The above mentioned penetrants were prepared by mixing the phospholipid(s) with a suitable membrane-softening agent, such as cholate or polysorbate, as the case may be, either in an aqueous buffer or in ethanol; occasionally chloroform was used. In the latter two cases, which gave similar results, the solvent was evaporated under vacuum (10 Pa, overnight). The resulting lipid film was then hydrated with a buffer (pH around 7) to get a 10 wt-% lipid suspension, by and large. Vesicles were brought to the final, desired size by sequential extrusion as described for liposomes, using mainly filters with smaller pore sizes. The final size of Transfersomes was similar to that of liposomes.

[0145] Changing the surfactant-to-lipid ratio is believed to affect the mixed lipid bilayers deformability: the higher the surfactant concentration, the more adaptable is the resulting aggregate, up to the concentration at which the mixed lipid membranes became unstable, owing to the high surfactant concentration. At such point the mixed aggregates revert into micelles which no longer change their shape easily, owing to the low compressibility of the micelle interior. Vesicles without a surfactant or some other edge active ingredient, which are commonly known as liposomes and have at least 10x less flexible membranes than the more deformable mixed lipid vesicles, are a convenient negative control for the latter. The other obvious control are

[0146] ***Mixed lipid micelles*** containing similar ingredients as the corresponding highly adaptable penetrants, but in a different ratio, such that the edge active component (typically, but not necessarily, the surfactant) concentration is above the solubilization concentration value. To prepare said micelles, individual components were mixed in the aqueous phase and permitted them to interact until the mixture became optically clear, that is, solubilised, as judged by optical inspection or absorption measurement at 400 nm to 600 nm.

Experiments carried out on human volunteers

[0147] To test biological activity of insulin carriers in humans, a freshly prepared test formulation was used in the nose of two test subjects. The first was a normoglycaemic (male, 74 kg, 173 cm, 45 years); the second was a C-peptide negative IDDM patient (female, 62 kg, 167 cm, 26 years). The test persons fasted between 6 h and 12 h prior to insulin administration.

[0148] To follow the temporal variation of glucose concentration in the blood, 5 µL to 30 µ samples taken, every 10 min to 15 min, from the fingers on both arms. After an initial test period, during which the 'normal' blood glucose concentration and/or its change was determined, a suspension of carriers loaded with insulin (Transfersulin) was sprayed into each nostril, using conventional non-metered sprayer, in a series of 150 µL puffs. Care was taken to minimise the spill-over of test formulation into the throat or the dropping of said formulation from the nose.

[0149] Commercial glucometer (Accutrend™, Boehringer-Mannheim) was employed to determine the blood sugar concentration. At each time point, three individual, independent readings were made, except when the standard deviation was so high as to require repeated measurements.

[0150] The test formulations were made essentially as described in patent application PCT/EP98/06750. In brief, a suspension of highly adaptable penetrants with the above mentioned composition and an average diameter of the order of 100 nm to 150 nm was loaded with the drug, based on interfacial adsorption, and used within 24 h after the preparation. The drug-carrier association in the formulation was determined to be between 60 % and 70 %.

[0151] To administer the drug laden suspension into the nose, the preparation was filled into a commercial nebuliser (with a hand-driven pump, vertically oriented spraying nozzle and a puff volume of 150 µL, on the average). One puff was given into each nostril at a time, while the test subject gently sniffed.

[0152] The total number of puffs was a function of the application dose (in this case: 2). Immediate spill-over into the throat or partial leakage of the fluid from the nose was reported in 10-20 % of cases. No side effects, such as local irritation, sneezing, etc., were observed.

Example 1:

[0153]

28.4 mg/mL phosphatidylcholine from soy-bean
9.5 mg/mL phosphatidylglycerol from soy-bean
62.1 mg/mL Tween 80
phosphate buffer, pH 7.4
human recombinant (hr) insulin, 50 IU/mL
(from Actrapid 100 HM™, Novo-Nordisk)

Applied dose: ~5 IU per nostril

[0154] **Results** measured with a healthy subject are shown in figure 1. They reveal a transient decrease in the systemic blood glucose concentration after two administrations of the drug in carriers (closed symbols), with a maximum after 20-30 min and a return to the pretreatment value after approximately 1 h in either case. The observed change in glucose level corresponds to approximately 8.5 % of the decrease was measured in an independent experiment after intravenous injection of the drug (Inset: open symbols). The reproducibility remains to be improved, however, the first application, biased by the lack in administration skill having been less successful than the second administration.

[0155] No irritation or other unpleasant sensation was reported by the test person after nasal administration of insulin in highly adaptable penetrants.

Example 2:

Insulin loaded, highly adaptable carriers in an IDDM patient

[0156] highly adaptable penetrants:

as given in example 1

Applied dose: 25 IU per nostril

[0157] Test preparation and experiment was performed as described with previous example. The last administration of conventional insulin (Monotard™, Novo-Nordisk), at the dose of 22 IU was done at 10 p. m. on the previous day. Test subject, moreover, was stabilised by using long-acting insulin on the test day prior to nasal administration of the insulin associated with highly adaptable drug carriers.

[0158] **Results** of an experiment done with said IDDM patient is illustrated in figure 2. Owing to the lack of endogenic insulin production in this test subject, the pretreatment blood glucose concentration was slightly above the normal, but relatively constant. The change resulting from nasal drug administration with ultra-adaptable carriers, has more a step-like rather than a peak-like shape (closed symbols), completed within 75 min. This is precisely what one would expect for an IDDM patient. The result of an i.v. injection of rapidly acting insulin (Actrapid™, Novo-Nordisk) in the same test person on a different occasion (inset: open symbols) corroborates the conclusion. An estimate of apparent bioavailability of nasal insulin based on these data is around 4 % and, consequently, somewhat lower than that reported in example 1. This may have to do with the presumed variability in drug release between different formulations which is illustrated in the following examples.

[0159] Nasal administration of carrier-associated insulin, according to the test person, caused no adverse side effect, locally or systemically.

Examples 3-5:

Insulin associated with suboptimal carriers

[0160] Carriers
as in previous examples, but believed not to release the drug readily owing to the higher affinity of selected insulin batch for the carrier, which makes the drug adsorption irreversible.

Applied doses: 50 IU, 50 IU

[0161] **Results** of the test measurements done with several different vesicle suspensions, illustrated in figure 3, signal lack of action for the insulin administered nasally with such carriers. The blood glucose concentration in the investigated normoglycaemic test person remains the same before, during and after the drug administration, for several hours at least. This suggests that the mere presence of carriers, or their ingredients, is insufficient to improve the bioavailability of nasally applied macromolecules, such as insulin. To achieve the desired biological effect, the rate of drug release from the carrier must also be adequate, such rate being determined in in dedicated ex vivo experiments by using conventional protein binding deassociation techniques.

Animal experiments

Examples 6-9 :

Labelled insulin delivery across nasal mucosa of test mice

[0162] highly adaptable penetrants:

87.4 mg/mL phosphatidylcholine from soy bean (SPC)
12.6 mg/mL of a 50 % ionised cholic acid
phosphate buffer, 50 mM, pH 6.5
hr-Insulin (Actrapid™, Novo-Nordisk)
labelled insulin from Amersham
(345 µL contain 1.08 µg insulin and 1.725 mg BSA)

[0163] ^{125}I-labelled insulin (210 µL) was mixed with 210 µL of hr-insulin (Actrapid™ Novo-Nordisk, 100 HM) and purified 2 times by centrifugation to eliminate the non-bound label, which diffuses across the barrier much faster and better than whole drug molecules. 100 µL of the resulting solution was mixed with 150 µL phosphate buffer to yield pH around 7. Protein solution and lipids were processed together, bringing the final vesicle size by repeated extrusion through 100 nm pore filters to values around 150 nm.

[0164] Mice of NMRI strain (36 g to 51 g) from a local supplier were kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. Each mouse received 2.5 µL of labelled penetrant suspension containing insulin per nostril. Then, the decrease in total radioactivity was assessed by whole-body camera at least 2 times. At different times the mice were killed and all major organs were taken and measured separately. The carcass was measured in two steps, after organ elimination and then after separation of the head. Radioactivity in excrement and cage was also determined.

[0165] **Results** pertaining to different time-points are given in Figure 4. They show that substantial amount of nasally administered radioactivity is recovered from the body, even after exclusion of gastro-intestinal tract, especially during the first hours following suspension administration. Values in the blood are in the range of 9 % at 0.5 h and 2 %, the specific concentration falling from 3 %/mL at the beginning to 0.7 %/mL at the end. Activity

in the nose decreases from 10.4 % at 0.5 h to 0.3 % at 8 h. Liver values are between 2.3 % after 0.5 h, the maximum around 2.8 at 1 h and values above 1 % after 4 h. After 8 h, the residuum in the liver is around 0.4 %. The relatively high hepatic values are suggestive of passage of particles, that is, penetrants, through the barrier and subsequent uptake in the reticulo-endothelial system.

[0166] Corresponding CNS values are 0.1 % and 0.03 %. Maximum in the brain is measured between the first and second hour with app. 0.11 % and 0.14 %, respectively, which amounts to around 0.3 %/g organ. These, apparently low values compare favourably with the result of more conventional drug delivery into CNS which yields values below 0.5 % of injected dose or around 0.15 %/g organ, for example, when transferrin-receptor is used to deliver the drug (Pasechnik & Price, 1996). In the case of white-germ agglutinin 0.1 % was found in olfactory bulb.

Examples 10-11 :

[0167] highly adaptable penetrants

87.4 mg/mL phosphatidylcholine from soy bean (SPC)
12.6 mg/mL of a 50 % ionised cholic acid
phosphate buffer, 50 mM, pH 6.5
human recombinant insulin (Actrapid™, Novo-Nordisk)
labelled insulin from Amersham

[0168] In a related experiment, 345 µL of ^{125}I-labelled insulin was mixed with 345 µL of cold Actrapid™ (Novo-Nordisk) and purified 2 times, as in previous experiment. After addition of 200 µL phosphate buffer, 150 µL of resulting solution was mixed with the lipids and extruded to final vesicle size. The applied dose was 3 µL per nostril. Mice were killed after 1 h, fixed, cut in thin sections and inspected by the whole-body radiography. Free insulin in solution was used for comparison.

[0169] The results of above mentioned experiments (not shown) revealed high label accumulation in the nasal region, as one would expect, substantial spill-over into the GI tract, very high density in the bladder, but also some radioactivity in the liver, which appears to be slightly higher for the carrier-derived than for the free insulin.

Examples 12-13:

Labelled interferon-gamma delivery across nasal mucosa of test *mice*

[0170] highly adaptable penetrants

86.6 mg/mL phosphatidylcholine from soy bean (SPC)
13.4 mg/mL Na cholate
phosphate buffer, 10 mM, pH 7.2 (nominal)
1 mg IFN-gamma/mL suspension
(100 µCi/mL suspension, as 3-^{125}I-tyrosyl-IFN-gamma)

Applied dose: 5 µL nostril

[0171] Mice of NMRI strain (36±0.6 g) were housed and taken care of as described with previous examples. Prior to the test formulation application, the animals were sedated as described before. Test formulation then was administered through a fine catheter in two drops of 5 µL, resulting in the total dose of 1 mg lipid. After this, the animals were kept in separate cages to prevent mutual contamination.

[0172] Measured radioactivity in the blood was found to correspond to app. 2.5 % of the applied dose, liver concentration being at app. 2 % and colon concentration around 2.5 %, all after 2 h. The highest amount of radioactivity by then was recovered from the stomach (37 %) and in the cage plus excrement (32 %).

[0173] In the central nervous system (CNS) 0.06 % of total nasally applied dose, as judged by derived radioactivity, was present 1 h after the drug administration by means of highly adaptable, protein-loaded mixed lipid-surfactant vesicles.

Examples 14-19:

Cytokine delivery across the nasal mucosa of test mice

[0174] highly adaptable penetrants

37.7 mg/mL phosphatidylcholine from soy bean (SPC)
62.3 mg/mL polysorbate (Tween 80)
phosphate buffer, 10 mM, pH 6.5
Tetanus toxoid, as antigen (2 mg/mL)
Interferon-γ (IFG-γ)
Granulocyte-monocyte-colony stimulating factor (GM-CSF)
Interleukin 4 (IL-4)
Interleukin 12 (IL-12)

Applied dose: 3 µL per nostril

[0175] Mice of Swiss albino strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunisation. The antigen alone or in combination with various cytokines, both believed to be at least partly associated with the carriers, was positioned with a sequencing in front of the animal nose and left to be sucked-in by the latter. Blood samples were collected retro-orbitally and tested with specific antibodies directed against the employed antigen by measuring absorbance at 492 nm, after subtaction of blank samples with ELISA.

[0176] The results of above mentioned measurements, illustrated in figure 6, suggest that the presence of all tested cytokines in vaccination formulation, based on the highly adaptable antigen carriers, increases the serum absorbance compared to that characterising the non-modulated value, determined after simple immunocarrier administration. Relative differences are more likely consequences of diverse bio-potency of tested immuno-modulants employed in the present specific experimental system than indicative of variable macromolecular transport rate across the nasal mucosa.

[0177] The observed 100 % increase in serum absorbance measured for GM-CSF/IL-4 combination is remarkable, as it is known that neither polysorbate nor phosphatidylcholine ex soy-bean can markedly enhance permeation capability on their own. It is therefore reasonable to assume that the observed effect is not simply due to the delivery of antigen molecules (with the molar mass of 150 kDa) across the nasal mucosa but, moreover, testify that at least a proportion of co-administered cytokines has passed the barrier in a biologically active form.

Examples 20-21:

[0178] highly adaptable penetrants

as in examples 14-19, except for the absence of cytokines
Tetanus toxoid antigen (2 mg/mL)

[0179] Mixed lipid micelles

14.8 mg/mL phosphatidylcholine from soy bean (SPC)
85.2 mg/mL polysorbate (Tween 80)
phosphate buffer, 10 mM, pH 6.5
Tetanus toxoid antigen (2 mg/mL)

Applied dose: 3 µL per nostril

[0180] Experiments were done as described with previous examples (14-19).

[0181] Immune response in the animals treated with mixed lipid micelles as in Examples 14-19 was clearly inferior to that measured after the nasal application of antigen in the highly adaptable lipid vesicles, despite the fact that the latter contained a smaller amount of Tween 80 than the former. If the surfactant was responsible for the transport of macromolecules across nasal mucosa, owing to its action as skin permeation enhancer, precisely the opposite experimental outcome would have been expected.

[0182] This suggests that highly adaptable carriers (mixed lipid vesicles) transport macromolecules across the nasal mucosa by a mechanism other than the drug permeation.

Examples 22-29:

Aggregate size (stability) effect

[0183] Highly deformable vesicles with NaCh (Transfersomes™)

89.3 mg phosphatidylcholine from soy bean
10.7 mg sodium cholate (NaCh)
0.9 mL phosphate buffer, pH 6.5

[0184] (Mixed lipid) Micelles with NaCh, type 1

65 mg phosphatidylcholine from soy bean
35 mg sodium cholate
0.9mL phosphate buffer, pH 6.5

[0185] (Mixed lipid) Micelles with NaCh, type 2

31.6 mg phosphatidylcholine from soy bean
68.5 mg sodium cholate
0.9 mL phosphate buffer, pH 6.5

[0186] Highly deformable vesicles with Tw, Transfersomes™ type 1

37.7 mg phosphatidylcholine from soy bean
62.3 mg Tween 80 (Tw)
0.9 mL phosphate buffer, pH 6.5

[0187] Highly deformable vesicles with Tw ,Transfersomes™, type 2

64.5 mg phosphatidylcholine from soy bean
35.5 mg Tween 80
0.9 mL phosphate buffer, pH 6.5

[0188] (Mixed lipid) Micelles with Tw, type 1

13.2 mg phosphatidylcholine from soy bean
86.8 mg Tween 80
0.9 mL phosphate buffer, pH 6.5

[0189] (Mixed lipid) Micelles with Tw, type 2

7 mg phosphatidylcholine from soy bean
93 mg Tween 80
0.9 mL phosphate buffer, pH 6.5
0.10

[0190] Lipid vesicles (liposomes)

65 mg phosphatidylcholine from soy bean (SPC)
35 mg cholesterol
0.9 mL phosphate buffer, pH 6.5

[0191] Tetanus toxoid (2 mg/mL; home made) used at the dose of

40 µg (20 µL) TT per mouse and immunisation

[0192] The medium filtrate from a culture of *Clostridium tetani* grown in vitro was used as an purified antigen. Pure toxoid was purchased from Accurate Antibodies, NY, USA.

[0193] To test the effect of aggregate properties in the formulation, three kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) either comprising a flexible membrane (Transfersomes) or a relatively rigid membrane (liposomes) and much smaller micelles (diameter below 50 nm). The latter were chosen to mimick the more conventional approach of using detergents as nasal mucosa permeation enhancers.

[0194] Amongst the eight tested formulations, Transfersomes, on the average, give best results, but absolute titres are always very low, probably owing to the antigen impurity. Mixed lipid micelles are most efficient in creating IgA, but are not really different than the other aggregates in the case of IgG2a and IgM, whilst in the case of Ig2b they are comparable to Transfersomes. The IgG1 level, which is decisive for animal protection, is only significantly elevated when Transfersomes are used to deliver TT across through the nose (see figure 7a).

[0195] Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) are, relatively speaking, less efficient 'immuno-carriers' (see figure 7b), the more deformable Transfersomes with a higher Tw content standing clearly out in the case of IgG2a and IgM, are similar to the less deformable Transfersomes with a lower Tw content in the case IgG1 and IgG3, and are as efficient as mixed micelles with Tw in the case of IgA and IgG2b. The smallness of measured values is reason for the concern, however, which can best be overcome by using purer antigen.

[0196] Looking at the cumulative titre of all specific anti-TT antibodies in the serum, liposomes are relatively efficient 'immuno-carriers' in the primary and mature response (perhaps owing to the action of non-associated TT), whilst the Tw rich mixed micelles are the worst. NaCh Transfersomes are top performers in the late immune response (cf. figure 7c).

Examples 30-35:

Antigen dose and purity effect

[0197] Highly deformable vesicles (Transfersomes):

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
+/- 0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5

[0198] Tetanus toxoid (TT, from local source, purified by ultrafiltration)

0 µg, 40 µg or 80 µg TT/ mouse/ immunisation

[0199] To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times.

[0200] Dose dependence results are illustrated in figure 8a. The TT-specific increase in serum absorbance following TT administration through the nose by means of Transfersomes reveals a positive dose dependence in the primary and late immune response in the absence of LA, the presence of LA reverting this trend. Titre-wise and with regard to specific antibody isotype distribution, similar but not identical picture is obtained (cf. figures 8b and 8c). The survival data are indicative of good protection in every case. Taken together this suggests that the required dose for non-invasive nasal immunisation by means of highly deformable carriers is much lower than that required for a successful non-invasive TT administration through the skin.

[0201] Antigen purity effect. Comparison of the data shown in figure 8c and 7a and 7b shows that antigen purity strongly affects the level of murine immune response against tetanus toxin when the toxoid has been applied non-invasively through.

Examples 36-46 :

Route of administration

[0202] Highly deformable vesicles, NaCh Transfersomes™

as described with examples 1-8

[0203] Tetanus toxoid mixed with NaCh suspension

20 mg/mL sodium cholate in
phosphate buffer, pH 7

[0204] Tetanus toxoid dose: 40 µg TT per immunisation; 5 µg TT, 10 µg TT, 20 µg TT, 40 µg TT per immunisation.

[0205] Using the same experimental procedures as described with previous examples, the antibody-specific serum absorbance the corresponding antibody titre and isotype distribution, and the level of animal protection against tetanus toxin was determined after nasal, oral and subcutaneous antigen administration.

[0206] The results are given in figures 9. They reveal that the increase in serum absorbance, ultimately, is comparable after invasive and non-invasive antigen administration (figure 9a). However, the titre in the latter case is significantly lower except in the primary response. Interestingly, s.c. injection only produces superior results after the second boost, whereas the combination with TT and cholate, which then can act as nasal permeation enhancer in total antibody titre is better at

all times. The probable reason for this is the high concentration of IgG2b, as is seen from figure 9b. Injections elicit most efficiently the IgG1 and IgM type of antibodies.

[0207] Animals are well protected by any of above mentioned vaccinations with TT, but only after 2 boosts; in the case of nasal vaccination. In contrast, one boost is sufficient (data not shown). Using 4-8x lower doses of purified TT suffices for protection in the case of nasal vaccination, but not when the antigen is injected (cf. figure 10).

Example 47

Low molecular weight adjuvant (lipid A) effect

[0208] Highly deformable immuno-modulated TT-Transfersomes™:

as in examples 9-14

[0209] Tetanus toxoid: 2 mg/mL, with 20 µL or 40 µL corresponding to 40 µg or 80 µg TT per immunisation

[0210] It is believed that co-administration of immunoactive, typically immunopotentiating, molecules is advantageous for presentation TT associated with the carriers to the body. To substantiate this conclusion specifically the outcome of non-invasive immunopresentation of TT was compared by means of Transfersomes with or without monophosphoryl lipid A (LA), which is a well known immunostimulant known to elicit generation of TNF, for example. For the used, relatively high antigen doses no strong dependence was found, however. In either case substantial titres and a prophylactic immune response was reached, which was not the case with purified TT which profited from the presence of LA.

Examples 48-53:

High molecular weight immunomodulators (cytokines)

[0211] Highly deformable vesicles, Tw Transfersomes™:

as described with examples 1-8, plus
various cytokines (Interferon-γ, GM-CSF, IL-4, IL-12)
(0.05 mg IFN-γ; 0.004 mg GM-CSF; 0.004 mg IL-4 per mL, 0.004 mg IL-12 per mL)

[0212] Tetanus toxoid, 2 mg/mL, corresponding to 40 µg TT (purified, home prepared) per mouse/ immunisation

[0213] The effect of cytokines was studied individually and in combination. The results are given in figures 5. They suggest that GM-CSF plus IL-4 combination can support the generation of anti-TT antibodies in mice, as can, probably, IFN-γ and perhaps IL-12, and maybe IL-4 (cf. figure 11a). The strongest effect is seen in the case of IgM and IgA, except in the case of IL-12 usage, which only affects strongly IgG2b generation. The protection relevant IgG1 is increased strongly only by the combination of GM-CSF and IL-4, whereas IgG3 is not affected at all. Injection works best for IgG1 (cf. figure 11b).

Examples 54-58:

Combination of low and high molecular weight adjuvants (LA + IL-12) effect

[0214] Highly deformable vesicles, NaCh Transfersomes™,

as described with examples 1-8, plus
0.4 mg IL-12 per mL immunogen suspension
0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC

[0215] Tetanus toxoid (purified), 2 mg/mL, corresponding to 40 µg TT per mouse/ immunisation

[0216] The effect discussed with examples 25-31 was confirmed for a blend low molecular and high molecular weight immunoadjuvants. The results are given in figures 12 and show that the immunopotentiation by such a combination is especially strong during the early stage of immune response, the combination in any case being better than LA alone.

Examples 59-71:

Bacterial wall component, cholera toxin, as adjuvant:

[0217] Highly deformable vesicles, Transfersomes™ (Tfs):

TfsC
86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol
TfsT
36 mg phosphatidylcholine from soy bean (SPC)
64 mg Tween 80
0.9 mL phosphate buffer, 10 mM, pH 7
Cholera toxin (CT; Sigma, Neu-Ulm), 2 µg/immunisation, if specified, Tetanus toxoid (TT, pure; Accurate antibodies), 2 mg/mL.

[0218] Volume doses corresponding to 0 µg TT/ mouse/immunisation (negative control), 1 µg TT/ mouse, 5 µg TT/ mouse, 10 µg TT/ mouse, 20 µg TT/ mouse, 40 µg TT/mouse (in the absence of CT) and 0.5 µg TT/mouse/immunisation, 1 µg TT/mouse, 5 µg TT/ mouse (when using CT) was used intranasally in the type T Transfersomes (TfsT) in both nostrils and at the dose of 0.5 µg TT/mouse/immunisation in the type C

Transfersomes (TfsC) in 4-6 Swiss albino mice. Moreover, 20 µg TT/ mouse/immunisation in TfsT was injected subcutaneously at the corresponding site in the positive control group. Immunisations were done on days 1, 14, 28.

[0219] The protective effect of antigen applied in the nose was good when the antigen dose exceeded 20 µg/ immunisation; lower doses yielded insufficient, but detectable protection (cf. figure 13). When cholera toxin (CT) was included into the test formulation together with the tetanus toxoid, excellent protection was achieved already at the lowest of tested doses (0.5 µg/immunisation), independent of the ultra-deformable carrier composition. Protection was complete in all test groups containing CT in the formulation applied on the skin.

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Claims

1. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or forms of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounded by the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains as a carrier for the preparation of a pharmaceutical, preferably a vaccine composition, for transnasal administration.

2. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounded by the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, said penetrant being used in combination with a pharmaceutically active ingredient or an allergen or an antigen for the preparation of a transnasally administerable pharmaceutical composition for the treatment of infective diseases, endocrine disorders, preferably hypopituitarism, diabetes, hyperthyroidism, thyroiditis, most preferably Hashimoto's thyroiditis, subacute thyroiditis; adrenal disorders, preferably Addison's disease, secondary adrenal insufficiency, Cushing's syndrome; gastrointestinal disorders, preferably Crohn's disease, colitis; hemorrhagic diseases, preferably hemophilia, leukopenia, hypereosinophilic syndrome; musculoskeletal and connective tissue disorders, preferably rheumatoid arthritis, Sjögren's syndrome, Bechet's syndrome, lupus, scleroderma, polymyositis/dermatomyositis, polymyalgia rheumatica and temporal arthritis, polyarteriosis nodosa, Wegener's granulomatosis, mixed connective tissue disorder, ankylosing spondylitis, psoriatic arthritis; osteoarthritis, Paget's disease, sciatica, bursitis, tendonitis and tenosynovitis, epicondylitis, fibromyalgia, eosinophilic faciitis; neurological disorders, preferably pain, singultus, vertigo, seizure disorders, sleep disorders, transient ischemic attacks, spinal cord injury, demyelinating diseases, nerve root disorders, myasthenia gravis; oncological disorders; psychiatric disorders, preferably drug dependence, neuroses, mood disorders, schizophrenic disorders, delusional disorders; and/or for use in the field of gynecology, preferably for the treatment of dysmenorrhea, menopause, chronic anovulation, premature ovarian failure, endometriosis, infertility; and/or for use in the field of immunology, preferably transplant rejection, hyposensitation, allergen immunotherapy or prophylactic vaccination.

3. The use of claim 2 wherein the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an

antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an antiallergicum, antiarrhythmicum, antiarteroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, antidrepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum or antihypertonicum, an antihypotonicum, anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardiotonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flu agent, a haemostaticum, hypnoticum, an immunoglobuline or its fragment or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an antimigraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly) peptide (derivative), an opiate, an opthalmicum, (para)-sympaticomimeticum or (para)sympathicolyticum, a protein(derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an inhibitor (antagonist) or a promoter (agonist) of the activity of any of above mentioned agents or any combination of said active substances.

4. The use of claim 2 wherein the antigen is derived from a pathogen.

5. The use of claim 2 wherein said pathogen belongs to extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG),* particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. M. *tuberculosis)* and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases or to eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body which do not necessarily result from microbial infections.

6. The use of claim 2 wherein the antigen is used in a purified or even better in a pure form.

7. The use of claim 2 wherein the antigen is the antigenic determinant of hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases or else eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections.

8. The use of claim 2 wherein the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

9. The use of claim 2 wherein the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., a part of implantation material.

10. The use of any one of claims 1 and 2 and 3 to 9 additionally comprising a compound which releases or induces cytokine or anti-cytokine activity or exerts such an activity itself.

11. The use of claim 10 wherein the compound exerting cytokine activity is IL-4, IL-2 , TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, preferably IFN-alpha or IFN-β, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

12. The use of claim 10 wherein said compound with anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative, or an analogue thereof.

13. The use of claim 12 wherein the compound displaying or inducing cytokine or anti-cytokine activity and the pharmaceutically active ingredient or antigen or allergen are associated with the penetrant.

14. The use of any one of claims 1 to 13 wherein the less soluble self-aggregating molecule is a lipid, preferably a polar lipid, and the more soluble component is a surfactant or some more soluble form of the polar/basic lipid.

15. The use of any one of claims 1 to 14 wherein the more soluble component is an agent to be transported across the barrier, said agent having a tendency to form common large structures with the less soluble component(s) of the penetrant, typically in the form of a physical or a chemical complex.

16. The use of any one of claims 1 to 15 wherein the more soluble component tends to solubilise the penetrating droplet and is present in concentration not exceeding 99 mol% of the concentration required to disintegrate the droplet or, alternatively, not exceeding 99 mol% of the saturating concentration in the unsolubilised droplet, whichever is higher, values below 50 % of the former relative concentration being particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

17. The use of any one of claims 1 to 16 wherein the less soluble penetrant component is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of a lipid, preferably a polar lipid which is sufficiently soluble for the purpose of this invention.

18. The use of any one of claims 1 to 17 wherein the average penetrant diameter is between 25 nm and 500 nm, preferably between 30 nm and 250 nm, even more preferably between 35 nm and 200 nm and particularly preferably between 40 nm and 150 nm.

19. The use of any one of claims 1 to 18 wherein the penetrant concentration in the formulation for the use in human or animal nose is 0.001 to 20 weight-% of total dry mass in the formulation, in particular between 0.01 w-% and 15 w-%, more preferably between 0.1 w-% and 12.5 w-% and most preferred between 0.5 w-% and 10 w-%.

20. The use of any one of claims 1 to 19 wherein the supporting medium, e.g. a buffer, is selected to be a biocompatible solution with an osmotic activity similar to that of a monovalent electrolyte with concentration in the range between 1 mM and 500 mM, more preferably between 10 mM and 400 mM, even more preferably between 50 mM and 300 mM, and most preferably between 100 mM and 200 mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier.

21. The use of any one of claims 1 to 20 wherein the relative drug or agent concentration is between 0.001 and 40 weight-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.

22. The use of any one of claims 1 to 21 wherein the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.

23. The use of any one of claims 1 to 22 wherein the additives are included in the preparation to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc..

24. The use of any one of claims 1 to 23 wherein the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects.

25. The use of any one of claims 1 to 24 wherein the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

26. The use of any one of claims 1 to 25 wherein the efficiency of administration and the biological effects of the agent or drug chosen are controlled by using different application volumes.

27. The use of any one of claims 1 to 26 wherein said formulation is administered using a metered delivery device.

28. The use of any one of claims 1 to 27 wherein different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.

29. The use of any one of claims 1 to 28 wherein the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation administration in the nose.

30. The use of any one of claims 1 to 29 wherein the delivery device is loaded at the treatment site.

31. The use of any one of claims 1 to 30 wherein the device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.

32. The use of any one of claims 1 to 31 wherein the pharmaceutically active ingredient is for administration to the nervous system.

33. The use of claim 32 wherein the nervous system is the brain.

34. The use of any one of claims 1 to 33 wherein said pharmaceutical composition is a vaccine.

35. The use of claim 34 wherein the vaccine further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.

36. The use of claim 35 wherein said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus or rubella diseases.

37. The use of any one of claims 34 to 36 wherein said vaccine further comprises an adjuvant.

38. The use of claim 37 wherein said adjuvant is lipopolysaccharide, such as lipid A or a derivative or modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a microorganism; an extract of a microorganism, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, an LT halotoxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

39. The use of any one of claims 34 to 38 wherein said vaccine comprises a blend of MPL and IL-12 or GM-CSF and IL-4.

40. The use of any one of claims 34 to 39 wherein in said vaccine the relative immunogen/antigen dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen dose that would have to be injected to achieve the desired biological effect.

41. The use according to any one of claims 37 to 40 wherein in said vaccine the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than that used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

Patentansprüche

1. Verwendung eines Durchdringungsmittels, suspendiert oder dispergiert in einem Lösungsmittel, in der Form eines winzigen Flüssigkeitstropfens, der von einer membranähnlichen Hülle aus einer oder mehreren Schichten von mindestens zwei verschiedenen Stoffen oder zwei verschiedenen Formen eines Stoffes, mit der Tendenz zu aggregieren, umgeben ist, wobei die Stoffe oder Formen eines Stoffes sich mindestens um den Faktor 10 in der Löslichkeit in einem vorzugsweise wässrigen, flüssigen Medium unterscheiden, so dass der mittlere Durchmesser von Homoaggregaten des löslicheren Stoffes oder Form des Stoffes oder der mittlere Durchmesser der Heteroaggregate bestehend aus beiden Stoffen oder Formen des Stoffes kleiner ist als der mittlere

Durchmesser der Homoaggregate des weniger löslichen Stoffes oder Form des Stoffes, und/oder wobei die löslichere Komponente die Tendenz hat, den durchdringenden Tropfen zu solubilisieren, und wobei der Gehalt einer solchen Komponente bis zu 99 mol-% der Konzentration beträgt, die zum Solubilisieren des Tropfen erforderlich ist, oder ansonsten bis zu 99 mol-% der Sättigungskonzentration in dem nicht solubilisierten Tropfen entspricht, was auch immer höher ist, und/oder wobei die elastische Deformationsenergie des von der membranähnlichen Hülle umgebenen Tropfens mindestens fünffach niedriger ist, vorzugsweise mindestens zehnfach niedriger und idealerweise mehr als zehnfach niedriger ist als diejenige von roten Blutzellen oder von Phospholipid-Doppelschichten mit flüssigen, aliphatischen Ketten, als Träger zur Herstellung eines Arzneimittels, vorzugsweise eines Impfstoffes, zur transnasalen Verabreichung.

2. Verwendung eines Durchdringungsmittels, suspendiert oder dispergiert in einem Lösungsmittel, in der Form eines winzigen Flüssigkeitstropfens, der von einer membranähnlichen Hülle aus einer oder mehreren Schichten von mindestens zwei verschiedenen Stoffen oder zwei verschiedenen Formen eines Stoffes, mit der Tendenz zu aggregieren, umgeben ist, wobei die Stoffe oder Formen eines Stoffes sich mindestens um den Faktor 10 in der Löslichkeit in einem vorzugsweise wässrigen, flüssigen Medium unterscheiden, so dass der mittlere Durchmesser von Homoaggregaten des löslicheren Stoffes oder Form des Stoffes oder der mittlere Durchmesser der Heteroaggregate bestehend aus beiden Stoffen oder Formen des Stoffes kleiner ist als der mittlere Durchmesser der Homoaggregate des weniger löslichen Stoffes oder Form des Stoffes, und/oder wobei die löslichere Komponente die Tendenz hat, den durchdringenden Tropfen zu solubilisieren, und wobei der Gehalt einer solchen Komponente bis zu 99 mol-% der Konzentration beträgt, die zum Solubilisieren des Tropfen erforderlich ist, oder ansonsten bis zu 99 mol-% der Sättigungskonzentration in dem nicht solubilisierten Tropfen entspricht, was auch immer höher ist, und/oder wobei die elastische Deformationsenergie des von der membranähnlichen Hülle umgebenen Tropfens mindestens fünffach niedriger ist, vorzugsweise mindestens zehnfach niedriger und idealerweise mehr als zehnfach niedriger ist als diejenige von roten Blutzellen oder von Phospholipid-Doppelschichten mit flüssigen, aliphatischen Ketten, wobei das Durchdringungsmittel in Verbindung mit einem pharmazeutisch aktiven Bestandteil oder einem Allergen oder einem Antigen verwendet wird zur Herstellung eines transnasal verabreichbaren Arzneimittels zur Behandlung von infektiösen Krankheiten, endokrinen Störungen, vorzugsweise Hypophysenvorderlappeninsuffizienz, Diabetes, Schilddrüsenüberfunktion, Schilddrüsenentzündung, vorzugsweise Hashimoto's Schilddrüsenentzündung, subakute Schilddrüsenentzündung; Nebennierenstörungen, vorzugsweise Addison's Krankheit, sekundäre Nebenniereninsuffizienz, Cushing's Syndrom; gastrointestinalen Störungen, vorzugsweise Crohn's-Krankheit, Colitis; hämorrhagischen Krankheiten, vorzugsweise Hämophilie, Leukopenie, hypereosinophiles Syndrom; Skelettmuskelstörungen und Störungen des Bindegewebes, vorzugsweise rheumatoide Arthritis, Sjögren's Syndrom, Bechet's Syndrom, Lupus, Scleroderma, Polymyositis/Dermatomyositis, Polymyalgia rheumatica und temporäre Arthritis, Polyarteriosis nodosa, Wegener's Granulomatosis, kombinierte Störung des Bindegewebes, Spondylitis ankylosans, psoriatische Arthritis, Osteoarthritis, Paget's Krankheit, Ischiassyndrom, Bursitis, Tendonitis und Tenosynovitis, Epicondylitis, Fibromyalgie, eosinophile Faciitis; neurologische Störungen, vorzugsweise Schmerz, Singultus, Vertigo, Krampfanfall-auslösende Erkrankungen, Schlafstörungen, transiente ischämische Anfälle, Verletzungen des Rückenmarks, demyelinisierende Krankheiten, Nervenwurzelkrankheiten, Myasthenia gravis; onkologische Krankheiten; psychiatrische Krankheiten, vorzugsweise Medikamentenabhängigkeit, Neurosen, Stimmungsstörungen, schizophrene Krankheiten, Paranoia; und/oder zur Verwendung in der Gynäkologie, vorzugsweise zur Behandlung von Dysmenorrhoe, Menopause, chronische Anovulation, vorzeitige Ovarialinsuffizienz, Endometriose, Unfruchtbarkeit; und/oder zur Verwendung in der Immunologie, vorzugsweise Abstoßung von Transplantaten, Hyposensibilisierung, Allergenimmuntherapie oder prophylaktische Impfung.

3. Verwendung nach Anspruch 2, wobei der pharmazeutisch aktive Bestandteil ein Adrenocorticostatikum, ein Adrenolytikum, ein Androgen oder Antiandrogen, ein Antiparasitikum, ein Anabolikum, ein Anaesthetikum oder Analgetikum, ein Analeptikum, ein Antiallergikum, Antiarrhythmikum, Antiarterosklerotikum, Antiasthmatikum, und/oder Bronchospasmolytikum, ein Antibiotikum, Antidepressivum und/oder Antispsychotikum, ein Antidiabetikum, ein Antidot, ein Antiemetikum, ein Antiepileptikum, Antifibrinolytikum, Antikonvulsivum oder Anticholinergikum, ein Enzym, ein Coenzym oder der entsprechende Enzyminhibitor, ein Antihistaminikum oder Antihypertonikum, ein Antihypotonikum, Antikoagulans, Antimykotikum, Antimyasthenikum, ein Mittel gegen Morbus Alzheimer oder Morbus Parkinson, ein Antiphlogistikum, Antipyretikum, Antirheumatikum, Antiseptikum, ein respiratorisches Analeptikum, oder ein respiratorisches Stimulans, ein Broncholytikum, Kardiotonikum, Chemothera-

peutikum, Koronardilatator, ein Cytostatikum, ein Diuretikum, ein Ganglium-Blocker, ein Glucocorticoid, ein Antigrippemittel, ein Haemostatikum, Hypnotikum, ein Immunglobulin oder sein Fragment oder jeder andere immunologisch aktive Stoff, wie ein Immunmodulator, ein bioaktives Kohlenhydrat (Derivat), ein Kontrazeptivum, ein Antimigränemittel, ein Corticosteroid, ein Muskelrelaxans, ein Narkotikum, ein Neurotherapeutikum, ein (Poly)nukleotid, ein Neuroleptikum, ein Neurotransmitter, ein (Poly)peptid(derivat), ein Opiat, ein Opthalmicum, (Para)sympaticomimetikum, oder (Para)sympaticolythikum, ein Protein(derivat), ein Psoriasis/Neurodermitis-Wirkstoff, ein Mydriatikum, ein Psychostimulans, Rhinologikum, ein schlafinduzierendes Mittel, ein Beruhigungsmittel, ein Spasmolytikum, Tuberkulostatikum, Urologikum, ein Vasoconstrictor oder Vasodilatator, ein Virostatikum, ein wundheilender Stoff, ein Inhibitor (Antagonist) oder ein Promotor (Agonist) der Aktivität jeder der oben angeführten Agentien oder jede Kombination der aktiven Stoffe.

4. Verwendung nach Anspruch 2, wobei das Antigen von einem Pathogen stammt.

5. Verwendung nach Anspruch 2, wobei das Pathogen zu extrazellulären Bakterien gehört, einschließlich eiterbildenden Kokken, wie *Staphylococcus* und *Streptococcus*, gram-negativen Bakterien, wie Meningococcus- und Gonococcus-Arten, *Neisseria-Arten*, gram-negativen Bakterien, einschließlich Darmorganismen wie *E. coli, Salmonella, Shigella, Pseudomonas, Diphteria, Bordetella Pertussis,* und gram-positiven Bakterien (z.B. *Bacillus pestis, BCG*), besonders anaeroben, wie die Clostridium-Arten, Bakterien und Viren, die innerhalb von Wirtszellen überleben und sich replizieren, umfassend Mycobakterien (z.B. *M. tuberculosis)* und *Listeria monocytogenes,* Retroviren und Adenoviren, einschließlich Hepatitisvirus, (menschlichen) Immunschwächevirus, Herpesviren, Pocken (Windpokken)-, Influenza-, Masem-, Mumps- und Polio-Viren, Cytomegalievirus, Rhinovirus, usw., und Pilze, die innerhalb von Wirtszellen gedeihen, einem Parasiten, einschließlich tierischer Parasiten, wie Protozoen und Helminthen, und Ectoparasiten, wie Zecken und Milben, oder *Brucella-Arten,* einschließlich des Cholera verursachenden Agens, Haemophilus-Arten, ebenso wie Pathogene, die Parathyphus, Pest, Tollwut, Tetanus und Röteln auslösen, und Pathogene, die verschiedene Neoplasien, Autoimmunkrankheiten oder die mit anderen pathologischen Zuständen des tierischen oder menschlichen Körpers verwandt sind, verursachen, die nicht notwendigerweise von pathogenen Infektionen herrühren.

6. Verwendung nach Anspruch 2, wobei das Antigen in gereinigter oder noch besser in einer reinen Form verwendet wird.

7. Verwendung nach Anspruch 2, wobei das Antigen die antigene Determinante vom Hepatitisvirus, (menschlichem) Immunschwächevirus, Herpesviren, Pocken (Windpocken)-, Influenza-, Masern-, Mumps- und Polio-Viren, Cytomegalievirus, Rhinovirus, usw. und Pilzen, die innerhalb von Wirtszellen gedeihen, einem Parasiten, einschließlich tierischer Parasiten, wie Protozoen und Helminthen, und Ectoparasiten, wie Zecken und Milben, oder *Brucella*-Arten, einschließlich des Cholera verursachenden Agens, Haemophilus-Arten, ebenso wie Pathogenen, die Parathyphus, Pest, Tollwut, Tetanus und Röteln auslösen, und Pathogenen, die verschiedene Neoplasien, Autoimmunkrankheiten oder die mit anderen pathologischen Zuständen des tierischen oder menschlichen Körpers verwandt sind, verursachen, die nicht notwendigerweise von pathogenen Infektionen herrühren, ist.

8. Verwendung nach Anspruch 2, wobei das Allergen xenogenen oder endogenen Ursprungs ist, von einem Mikroorganismus, einem Tier oder einer Pflanze stammt, oder zu der Gruppe künstlicher und/oder reizender anorganischer Stoffe gehört, oder zu solchen Teilen oder Komponenten des menschlichen Körpers gehören, die fälschlicherweise durch das Körperimmunsystem prozessiert oder dem Körperimmunsystem ausgesetzt wurden.

9. Verwendung nach Anspruch 2, wobei das Allergen zu der Klasse von Inhalationsallergenen gehört, einschließlich, aber nicht beschränkt auf verschiedene Pollen, Sporen, Stückchen von Tierhaar, Haut, Feder, natürlichen und synthetischen Textilien, Weizen, (Haus)-Staub, einschließlich Milben; ferner Nahrungsmittel- und Medikamentenallergene; Kontaktallergene; Injektions-, Invasions- oder Depotallergene, wie verschiedene (gastrointestinale) Würmer, Echinokokken, Trichinen, usw., einen Teil eines Implantationsmaterials.

10. Verwendung nach einem der Ansprüche 1, 2 und 3 bis 9, zusätzlich umfassend eine Verbindung, die Cytokin- oder Anti-Cytokin-Aktivität freisetzt oder induziert oder selbst solch eine Aktivität aufweist.

11. Verwendung nach Anspruch 10, wobei die Verbindung, die Cytokin-Aktivität aufweist, IL-4, IL-2, TGF, IL-6, TNF, IL-1α und IL-1β, ein Typ I-Interferon, vorzugsweise IFN-α oder IFN-β, IL-12, IFN-γ, TNF-β, IL-5 oder IL-10 ist.

12. Verwendung nach Anspruch 10, wobei die Verbindung mit Anti-Cytokin-Aktivität ein Anti-Cytokin-An-

tikörper oder das entsprechende aktive Fragment, ein Derivat oder ein Analog davon ist.

13. Verwendung nach Anspruch 12, wobei die Verbindung, die Cytokin- oder Anti-Cytokin-Aktivität aufweist oder induziert, und der pharmazeutisch aktive Bestandteil oder Antigen oder Allergen mit dem Durchdringungsmittel verbunden sind.

14. Verwendung nach einem der Ansprüche 1 bis 13, wobei das weniger lösliche, selbst aggregierende Molekül ein Lipid, vorzugsweise ein polares Lipid ist, und die löslichere Komponente ein grenzflächenaktiver Stoff oder eine etwas löslichere Form des polaren/basischen Lipids ist.

15. Verwendung nach einem der Ansprüche 1 bis 14, wobei die löslichere Komponente ein über das Hindernis zu transportierendes Agens ist, wobei das Agens eine Tendenz hat, gemeinsame, große Strukturen mit der weniger löslicheren Komponente (n) des Durchdringungsmittels zu bilden, typischerweise in Form eines physikalischen oder chemischen Komplexes.

16. Verwendung nach einem der Ansprüche 1 bis 15, wobei die löslichere Komponente dazu tendiert, den durchdringenden Tropfen zu solubilisieren und in einer Konzentration vorliegt, die 99 mol% der Konzentration, die nötig ist, den Tropfen aufzulösen, nicht übersteigt, oder alternativ 99 mol% der Sättigungskonzentration in dem ungelösten Tropfen nicht übersteigt, welche auch immer höher ist, wobei Werte unter 50% der ersteren relativen Konzentration besonders geeignet sind, mit Werten unter 40 Rel-% oder sogar um und unter 30 Rel-% noch geeigneter sind, wobei im Fall, dass Tropfen, die nicht durch die löslichere Komponente solubilisiert werden können, relative Konzentrationen am bevorzugtesten sind, die die oben genannten relativen Konzentrationen um den Faktor bis 2 übersteigen.

17. Verwendung nach einem der Ansprüche 1 bis 16, wobei die weniger lösliche, durchdringende Komponente ein polares Lipid, und die löslichere Komponente ein grenzflächenaktiver Stoff oder ein grenzflächenaktives Stoff-ähnliches Molekül ist oder ansonsten diejenige Form eines Lipides, vorzugsweise eines polaren Lipides, die genügend löslich für den Zweck dieser Erfindung sind.

18. Verwendung nach einem der Ansprüche 1 bis 17, wobei der mittlere Durchmesser des Durchdringungsmittels zwischen 25 nm und 500 nm, vorzugsweise zwischen 30 nm und 250 nm, bevorzugt zwischen 35 nm und 200 nm und besonders bevorzugt zwischen 40 nm und 150 nm ist.

19. Verwendung nach einem der Ansprüche 1 bis 18, wobei die Konzentration des Durchdringungsmittels in der Formulierung zur Verwendung in der menschlichen oder tierischen Nase 0,001 bis 20 Gewichtsprozent der Gesamttrockenmasse in der Formulierung, im besonderen zwischen 0,01 Gewichtsprozent und 15 Gewichtsprozent, bevorzugt zwischen 0,1 Gewichtsprozent und 12,5 Gewichtsprozent und am meisten bevorzugt zwischen 0,5 Gewichtsprozent und 10 Gewichtsprozent, ist.

20. Verwendung nach einem der Ansprüche 1 bis 19, wobei das unterstützende Medium, z.B. ein Puffer, ausgewählt ist als biokompatible Lösung mit einer osmotischen Aktivität ähnlich der eines einwertigen Elektrolyten mit einer Konzentration im Bereich zwischen 1 mM und 500 mM, bevorzugt zwischen 10 mM und 400 mM, mehr bevorzugt zwischen 50 mM und 300 mM und am meisten bevorzugt zwischen 100 mM und 200 mM ist oder sonst solch eine Lösung, die in der Praxis genügend Durchdringungsstabilität kombiniert mit genügender Transportrate über das Hindernis gewährleistet.

21. Verwendung nach einem der Ansprüche 1 bis 20, wobei die relative Konzentration des Wirkstoffes oder Mittels zwischen 0,001 und 40 Gewichtsprozent der gesamten Durchdringungsmittel-Masse, insbesondere zwischen 0,01 Gewichtsprozent und 30 Gewichtsprozent, besser zwischen 0,1 Gewichtsprozent und 25 Gewichtsprozent und am meisten bevorzugt zwischen 0,5 und 15 Gewichtsprozent ist.

22. Verwendung nach einem der Ansprüche 1 bis 21, wobei das die Wirkstoffe und Träger unterstützende Medium ein biokompatibler Puffer mit einem pH-Wert zwischen 4 und 10, häufiger zwischen 5 und 9 und am häufigsten zwischen 6 und 8 ist.

23. Verwendung nach einem der Ansprüche 1 bis 22, wobei die Zusätze im Arzneimittel enthalten sind, um die Systemsensitivität gegenüber chemischem, biologischem oder Umgebungs-Stress zu verringern, einschließlich Antioxidantien, Antagonisten unerwünschter Enzymaktivität, Gefrierschutzzusätze, Antibiotika, usw., oder sonstige Modulatoren von physikalisch wichtigen Systemeigenschaften, wie Formulierungsviskosität, usw.

24. Verwendung nach einem der Ansprüche 1 bis 23, wobei die relative Wirkstoffdosis oder Mitteldosis, die nicht-invasiv durch die Nase mittels eines sehr anpassungsfähigen Trägers verabreicht werden soll, so gewählt ist, dass sie sich zwischen 0,1 und 500-fach, öfters zwischen 0,5-fach und 250-fach, und bevorzugter zwischen 1-fach und 100-fach von der entsprechenden Arzneistoffoder Mitteldosis un-

terscheidet, die hätte injiziert werden müssen, um die gewünschten biologischen Effekte zu erreichen.

25. Verwendung nach einem der Ansprüche 1 bis 24, wobei die verabreichte Dosis des Durchdringungsmittels zwischen 0,01 mg und 15 mg pro Nasenloch, öfters im Bereich 0,1 und 10 mg pro Nasenloch und vorzugsweise zwischen 0,5 mg und 5 mg pro Nasenloch ist.

26. Verwendung nach einem der Ansprüche 1 bis 25, wobei die Effizienz der Verabreichung und die biologischen Effekte des gewählten Mittels oder Wirkstoffs durch die Verwendung verschiedener Verabreichungsvolumina kontrolliert wird.

27. Verwendung nach einem der Ansprüche 1 bis 26, wobei die Formulierung unter Verwendung einer kalibrierten Verabreichungsvorrichtung verabreicht wird.

28. Verwendung nach einem der Ansprüche 1 bis 27, wobei verschiedene Verabreichungsvolumina gewählt sind, um die Effizienz der Verabreichung und die biologischen Effekte des gewählten Mittels oder Wirkstoffs zu kontrollieren.

29. Verwendung nach einem der Ansprüche 1 bis 28, wobei die Durchdringungsmittel in Suspension mit den Wirkstoffen oder Mitteln innerhalb von 24 Stunden vor der Verabreichung der Formulierung beladen werden, vorzugsweise 360 Minuten, bevorzugter 60 Minuten und am meisten bevorzugt 30 Minuten vor der Verabreichung der erhaltenen Formulierung in die Nase.

30. Verwendung nach einem der Ansprüche 1 bis 29, wobei die Verabreichungsvorrichtung an der Stelle der Behandlung beladen wird.

31. Verwendung nach einem der Ansprüche 1 bis 30, wobei die Vorrichtung getrennt mit Durchdringungsmitteln und den Molekülen, besonders biologischen Agenzien, die mit diesen verbunden werden sollen, beladen wird.

32. Verwendung nach einem der Ansprüche 1 bis 31, wobei der pharmazeutisch aktive Bestandteil zur Verabreichung an das Nervensystem ist.

33. Verwendung nach Anspruch 32, wobei das Nervensystem das Gehirn ist.

34. Verwendung nach einem der Ansprüche 1 bis 33, wobei das Arzneimittel ein Impfstoff ist.

35. Verwendung nach Anspruch 34, wobei der Impfstoff weiterhin einen Pathogenextrakt oder eine Verbindung eines Pathogens oder ein Fragment oder ein Derivat davon umfasst.

36. Verwendung nach Anspruch 35, wobei der Pathogenextrakt oder die Verbindung ausgewählt ist aus Hepatitisvirus, (menschlichem) Immunschwächevirus, Herpesviren, Pocken (Windpocken)-, Influenza-, Masern-, Mumps- oder Polio-Viren, Cytomegalievirus, Rhinovirus, usw., und Pilzen, die innerhalb von Wirtszellen gedeihen, einem Parasiten, einschließlich tierischer Parasiten, wie Protozoen und Helminthen, und Ectoparasiten, wie Zecken und Milben, oder *Brucella*-Arten, einschließlich des Cholera verursachenden Agens, Haemophilus-Arten, ebenso wie Pathogenen, die Parathyphus, Pest, Tollwut, Tetanus oder Röteln auslösen.

37. Verwendung nach einem der Ansprüche 34 bis 36, wobei der Impfstoff weiterhin ein Adjuvans umfasst.

38. Verwendung nach Anspruch 37, wobei das Adjuvans ein Lipopolysaccharid ist, wie Lipid A oder ein Derivat oder eine Modifikation davon, wie Monophosphoryllipid A, oder sein Analog, wie ein Fettderivat von Saccharose, Cordfaktor (Trehalose-Dimycolat), Muramyldipeptid, oder ein anderes (Poly) saccharid oder (Poly)peptid identisch mit oder ähnlich einem immunologisch aktiven Teil einer Membran eines Mikroorganismus ist; ein Extrakt eines Mikroorganismus, einschließlich bakterieller Exo- und Endotoxine, vorzugsweise Choleratoxin und das hitzelabile Toxin von *E. coli,* ein A-Kette-Derivat, eine Komponente mit einer ADP-ribosylierenden Aktivität, ein Peptidoglycan, ein von Clostridium stammendes Toxin, ein LT-Halotoxin, gereinigtes Proteinderivat *von M. tuberculosis,* LT-R192G, Fibronectinbindendes Protein I von *Streptococcus pyrogenes,* oder ein äußeres Membranprotein von Gruppe B-*Neisseria meningititdis* (GBOMP).

39. Verwendung nach einem der Ansprüche 34 bis 38, wobei der Impfstoff eine Mischung von MPL und IL-12 oder GM-CSF und IL-4 umfasst.

40. Verwendung nach einem der Ansprüche 34 bis 39, wobei im Impfstoff die mittels sehr anpassungsfähiger Träger nicht-invasiv durch die Nase zu verarbreichende relative Immunogen/Antigen-Dosis so gewählt ist, dass sie sich zwischen 0,01-fach und 100-fach, öfters zwischen 0,05-fach und 75-fach, und bevorzugt zwischen 0,1-fach und 50-fach von der entsprechenden Immunogen/Antigen-Dosis unterscheidet, die hätte injiziert werden müssen, um den gewünschten biologischen Effekt zu erreichen.

41. Verwendung nach einem der Ansprüche 37 bis 40, wobei im Impfstoff die Konzentration des transnasal

verabreichten Adjuvans zwischen 10-fach niedriger und bis zu 1000-fach höher ist als die, die mit den entsprechenden subkutan injizierten Formulierungen unter Verwendung ähnlicher Antigene verwendet wird, wobei die transnasal verabreichte Immunadjuvans-Konzentration sich öfters von der injizierten Immunadjuvans-Kontentration um den Faktor zwischen 0,5 und 100 unterscheidet, oder besser, durch den Faktor zwischen 1 und 50, und am besten zwischen 2 und 25.

Revendications

1. Utilisation d'un agent pénétrant en suspension ou dispersé dans un solvant sous la forme d'une minuscule gouttelette de fluide entourée par un enrobage semblable à une membrane fait d'une ou plusieurs couches d'au moins deux substances différentes ou de deux formes différentes d'une substance ayant tendance à s'agréger, lesdites substances ou formes de substance différant par au moins un facteur 10 en ce qui concerne leur solubilité dans un milieu liquide, de préférence aqueux, de sorte que le diamètre moyen des homo-agrégats de la substance ou forme de substance la plus soluble ou le diamètre moyen des hétéro-agrégats comprenant l'une et l'autre desdites substances ou formes de substance est plus petit que le diamètre moyen des homo-agrégats de la substance ou forme de substance la moins soluble et/ou dans laquelle le composant le plus soluble tend à solubiliser la gouttelette pénétrante et dans laquelle la teneur de ce composant va jusqu'à 99% molaires de la concentration nécessaire pour solubiliser la gouttelette ou bien correspond à jusqu'à 99% molaires de la concentration de saturation dans la gouttelette non solubilisée, à la plus grande des deux valeurs, et/ou dans laquelle l'énergie de déformation élastique de la gouttelette entourée par l'enrobage semblable à une membrane est au moins 5 fois moindre, de façon plus préférentielle au moins 10 fois moindre et idéalement plus de 10 fois moindre que celle des globules rouges ou des bicouches de phospholipides avec des chaînes aliphatiques fluides, comme véhicule pour la préparation d'un produit pharmaceutique, de préférence une composition de vaccin pour administration transnasale.

2. Utilisation d'un agent pénétrant en suspension ou dispersé dans un solvant sous la forme d'une minuscule gouttelette de fluide entourée par un enrobage semblable à une membrane fait d'une ou plusieurs couches d'au moins deux substances différentes ou de deux formes différentes d'une substance ayant tendance à s'agréger, lesdites substances ou formes de substance différant par au moins un facteur 10 en ce qui concerne leur solubilité dans un milieu liquide, de préférence aqueux, de sorte que le diamètre moyen des homo-agrégats de la substance ou forme de substance la plus soluble ou le diamètre moyen des hétéro-agrégats comprenant l'une et l'autre desdites substances ou formes de substance est plus petit que le diamètre moyen des homo-agrégats de la substance ou forme de substance la moins soluble et/ou dans laquelle le composant le plus soluble tend à solubiliser la gouttelette pénétrante et dans laquelle la teneur de ce composant va jusqu'à 99% molaires de la concentration nécessaire pour solubiliser la gouttelette ou bien correspond à jusqu'à 99% molaires de la concentration de saturation dans la gouttelette non solubilisée, à la plus grande des deux valeurs, et/ou dans laquelle l'énergie de déformation élastique de la gouttelette entourée par l'enrobage semblable à une membrane est au moins 5 fois moindre, de façon plus préférentielle au moins 10 fois moindre et idéalement plus de 10 fois moindre que celle des globules rouges ou des bicouches de phospholipides avec des chaînes aliphatiques fluides, ledit agent pénétrant étant utilisé en combinaison avec un principe actif pharmaceutique ou un allergène ou un antigène pour la préparation d'une composition pharmaceutique pouvant être administrée par voie transnasale pour le traitement de maladies infectieuses, de troubles endocriniens, de préférence l'hypopituitarisme, le diabète, l'hyperthyroïdisme, la thyroïdite, de la façon la plus préférentielle la thyroïdite de Hashimoto, la thyroïdite subaiguë ; des troubles surrénaux, de préférence la maladie d'Addison, l'insuffisance surrénale secondaire, le syndrome de Cushing ; des troubles gastro-intestinaux, de préférence l'iléite régionale, la colite ; des maladies hémorragiques, de préférence l'hémophilie, la leucopénie, le syndrome hyperéosinophile ; des troubles du tissu musculaire squelettique et conjonctif, de préférence la polyarthrite rhumatoïde, le syndrome de Sjogren, le syndrome de Béchet, le lupus, la sclérodermie, la polymyosite/dermatomyosite, la polymyalgie rhumatismale et l'artérite temporale, la polyartérite noueuse, la granulomatose de Wegener, le trouble mixte du tissu conjonctif, la spondylite ankylosante, l'arthrite psoriasique, l'ostéoarthrite, la maladie de Paget, la sciatique, la bursite, la tendinite et la ténosynovite, l'épicondylite, la fibromyalgie, la fasciite éosinophile ; des troubles neurologiques, de préférence la douleur, le hoquet, le vertige, les crises, les troubles du sommeil, les attaques ischémiques transitoires, les lésions de la colonne vertébrale, les maladies démyélinisantes, les troubles des racines nerveuses, la myasthénie gravis; des troubles oncologiques; des troubles psychiatriques, de préférence la dépendance vis-à-vis des drogues, les névroses, les troubles de l'humeur, les troubles schizophréniques, les troubles du délire ; et/ou pour une utilisa-

tion dans le domaine de la gynécologie, de préférence pour traiter la dysménorrhée, la ménopause, l'anovulation chronique ; l'insuffisance ovarienne précoce, l'endométriose, l'infertilité ; et/ou pour une utilisation dans le domaine de l'immunologie, de préférence le rejet de greffes, l'hyposensibilisation, l'immunothérapie allergénique ou la vaccination préventive.

3. Utilisation selon la revendication 2, dans laquelle le principe actif pharmaceutique est un adrénocorticostatique, un adrénolytique, un androgène ou antiandrogène, un antiparasitaire, un anabolisant, un anesthésique ou un analgésique, un analeptique, un anti-allergique, un anti-arythmique, un anti-artériosclérose, un anti-asthmatique et/ou un bronchospasmolytique, un antibiotique, un antidépresseur et/ou un antipsychotique, un antidiabétique, un antidote, un antiémétique, un anti-épileptique, un antifibrinolytique, un anticonvulsivant ou un anticholinergique, une enzyme, une coenzyme ou l'inhibiteur enzymatique correspondant, un antihistaminique ou un antihypertonique, un antihypotonique, un anticoagulant, un antimycotique, un antimyasthénique, un agent contre la maladie d'Alzheimer ou la maladie de Parkinson, un antiphlogistique, un antipyrétique, un antirhumatismal, un antiseptique, un analeptique respiratoire ou un stimulant respiratoire, un bronchiolytique, un cardiotonique, un agent chimiothérapeutique, un agent dilatant les coronaires, un cytostatique, un diurétique, un bloqueur des ganglions, un glucocorticoïde, un agent antigrippal, un hémostatique, hypnotique, une immunoglobuline ou fragment de celle-ci ou toute autre substance immunologiquement active telle qu'un immunomodulateur, un hydrate de carbone (dérivé) biologiquement actif, un contraceptif, un agent anti-migraineux, un corticostéroïde, un myorelaxant, un narcotique, un neurothérapeutique, un (poly)nucléotide, un neuroleptique, un neurotransmetteur, un (poly)peptide (dérivé), un opiat, un ophtalmique, un (para)sympathicomimétique ou un (para)sympathicolytique, une protéine (dérivé), un médicament contre le psoriasis/la névrodermite, un mydriatique, un psychostimulant, unagent rhinologique, un agent induisant le sommeil, un agent sédatif, un spasmolytique, un tuberculostatique, un agent urologique, un vasoconstricteur ou un vasodilatateur, un virustatique, une substance cicatrisante, un inhibiteur (antagoniste) ou un promoteur (agoniste) de l'activité de l'un quelconque des agents précités ou des combinaisons quelconques desdites substances actives.

4. Utilisation selon la revendication 2, dans laquelle l'antigène est dérivé d'un agent pathogène.

5. Utilisation selon la revendication 2, dans laquelle ledit agent pathogène appartient au groupe des bactéries extracellulaires, comprenant des coccus pyogènes tels que *Staphylococcus* et *Streptococcus*, des bactéries Gram négatives telles que les espèces *Meningococcus* et *Gonococcus*, des espèces de *Neisseria*, des bactéries Gram négatives comprenant des micro-organismes entériques tels que *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella pertussis*, et des bactéries Gram positives (par exemple *Bacillus pestis*, BCG), en particulier des anaérobies tels que les espèces *Clostridium*, des bactéries et des virus qui survivent et se répliquent dans des cellules hôtes, comprenant des mycobactéries (telles que *M. tuberculosis*) et *Listeria monocytogenes*, des rétrovirus et des adénovirus comprenant le virus de l'hépatite, le virus de l'immunodéficience (humaine), les herpèsvirus, le virus de la variole (varicelle), les virus de la grippe, de la rougeole, des oreillons et de la poliomyélite, le cytomégalovirus, le rhinovirus, etc. et des champignons qui prospèrent à l'intérieur de cellules hôtes, un parasite, comprenant des parasites des animaux tels que les protozoaires et les helminthes, et des ectoparasites tels que les tiques et les acariens, ou les espèces *Brucella*, comprenant l'agent responsable du choléra, les espèces *Haemophilus*, ainsi que des agents pathogènes qui déclenchent la fièvre paratyphoïde, la peste, la rage, le tétanos et rubéole, ou bien des cellules eucaryotes ou des parties de celles-ci qui provoquent diverses néoplasies, maladies auto-immunes et autres états pathologiques de l'organisme animal ou humain qui ne sont pas dues nécessairement à des infections microbiennes.

6. Utilisation selon la revendication 2, dans laquelle l'antigène est utilisé sous une forme purifiée ou mieux encore sous forme pure.

7. Utilisation selon la revendication 2, dans laquelle l'antigène est le déterminant antigénique du virus de l'hépatite, du virus de l'immunodéficience (humaine), des herpèsvirus, des virus de la variole (varicelle), de la grippe, de la rougeole, des oreillons et de la poliomyélite, du cytomégalovirus, du rhinovirus, etc., et de champignon qui prospèrent à l'intérieur de cellules hôtes, d'un parasite, comprenant des parasites des animaux tels que les protozoaires et les helminthes, et d'ectoparasites tels que les tiques et les acariens, ou de l'espèce *Brucella*, comprenant l'agent responsable du choléra, de l'espèce *Haemophilus*, ainsi que des agents pathogènes qui déclenchent la fièvre paratyphoïde, la peste, la rage, le tétanos et la rubéole, ou bien des cellules eucaryotes ou des parties de celles-ci qui provoquent diverses néoplasies, maladies auto-immunes et autres états pathologiques de l'organisme animal ou humain qui ne sont pas dues nécessai-

rement à des infections microbiennes.

8. Utilisation selon la revendication 2, dans laquelle l'allergène est d'origine allogène ou endogène, dérivé d'un micro-organisme, d'un animal ou d'une plante, ou appartient au groupe des substances inorganiques fabriquées par l'homme et/ou irritantes, ou à des parties ou composants de l'organisme humain qui ont été incorrectement traités par le, ou exposés au, système immunitaire de l'organisme.

9. Utilisation selon la revendication 2, dans laquelle l'allergène appartient à la classe des allergènes par inhalation, comprenant de manière non limitative divers pollens, spores, morceaux de poils, peau et plumes d'animaux, textiles naturels et synthétiques, le blé, les poussières (domestiques) y compris les acariens ; également les allergènes alimentaires et médicamenteux ; les allergènes de contact ; les allergènes par injection, invasion ou dépôt tels que les différents vers (résidents dans le système gastro-intestinal), échinocoques, trichines, etc., une partie de matériau d'implant.

10. Utilisation selon l'une quelconque des revendications 1 et 2 et 3 à 9, comprenant en plus un composé qui libère ou induit une activité de cytokine ou anti-cytokine ou exerce lui-même une telle activité.

11. Utilisation selon la revendication 10, dans laquelle le composé qui exerce une activité de cytokine est l'IL-4, l'IL-2, le TGF, l'IL-6, le TNF, l'IL-1α et l'IL-1β, un interféron de type I, de préférence l'IFN-α ou l'IFN-β, l'IL-12, l'IFN-γ, le TNF-β, l'IL-5 ou l'IL-10.

12. Utilisation selon la revendication 10, dans laquelle ledit composé ayant une activité anti-cytokine est un anticorps anti-cytokine ou le fragment actif correspondant, un dérivé ou un analogue de celui-ci.

13. Utilisation selon la revendication 12, dans laquelle le composé qui présente ou induit une activité de cytokine ou anti-cytokine et le principe actif pharmaceutique ou l'antigène ou l'allergène sont associés à l'agent pénétrant.

14. Utilisation selon l'une quelconque des revendications 1 à 13, dans laquelle la molécule auto-agrégeante la moins soluble est un lipide, de préférence un lipide polaire, et le composant le plus soluble est un tensio-actif ou une forme plus soluble du lipide polaire/ basique.

15. Utilisation selon l'une quelconque des revendications 1 à 14, dans laquelle le composant le plus soluble est un agent destiné à être transporté à travers la barrière, ledit agent ayant tendance à former des structures communes de grande taille avec le ou les composants les moins solubles de l'agent pénétrant, typiquement sous la forme d'un complexe physique ou chimique.

16. Utilisation selon l'une quelconque des revendications 1 à 15, dans laquelle le composant le plus soluble tend à solubiliser la gouttelette pénétrante et est présent en une concentration non supérieure à 99% molaires de la concentration nécessaire pour désagréger la gouttelette ou, en variante, non supérieure à 99% molaires de la concentration de saturation dans la gouttelette non solubilisée, à la plus grande des deux valeurs, des valeurs en dessous de 50% de la première citée de ces concentrations relatives étant particulièrement utiles, des valeurs en dessous de 40% rel. ou même voisines et en dessous de 30% rel. étant encore plus avantageuses, tandis que, dans le cas de gouttelettes qui ne peuvent pas être solubilisées par le composant le plus soluble, des concentrations relatives qui dépassent les concentrations relatives précitées d'un facteur allant jusqu'à 2 sont les plus préférées.

17. Utilisation selon l'une quelconque des revendications 1 à 16, dans laquelle le composant le moins soluble de l'agent pénétrant est un lipide polaire et le composant le plus soluble est un tensio-actif ou une molécule semblable à un tensio-actif ou bien une forme de lipide, de préférence un lipide polaire, qui est suffisamment soluble pour les besoins de la présente invention.

18. Utilisation selon l'une quelconque des revendications 1 à 17, dans laquelle le diamètre moyen de l'agent pénétrant est compris entre 25 nm et 500 nm, de préférence entre 30 nm et 250 nm, de façon plus préférentielle entre 35 nm et 200 nm et de façon particulièrement préférée entre 40 nm et 150 nm.

19. Utilisation selon l'une quelconque des revendications 1 à 18, dans laquelle la concentration de l'agent pénétrant dans la formulation pour utilisation nasale chez l'homme ou l'animal est de 0,001 à 20% en poids de la masse sèche totale de la formulation, en particulier de 0,01% en poids à 15% en poids, de façon plus préférentielle de 0,1% en poids à 12,5% en poids et de la façon la plus préférentielle de 0,5% en poids à 10% en poids.

20. Utilisation selon l'une quelconque des revendications 1 à 19, dans laquelle le milieu porteur, par exemple un tampon, est choisi pour former une solution biocompatible ayant une activité osmotique similaire à celle d'un électrolyte monovalent à une concentration dans la plage de 1 mM à 500 mM, de façon plus préférentielle de 10 mM à 400 mM, de façon plus préférentielle encore de 50 mM à 300

mM et de la façon la plus préférentielle de 100 mM à 200 mM, ou bien une solution qui assure une stabilité suffisante en pratique de l'agent pénétrant combinée avec un taux de transport à travers la barrière suffisant en pratique.

21. Utilisation selon l'une quelconque des revendications 1 à 20, dans laquelle la concentration relative du médicament ou de l'agent est de 0,001 à 40% en poids de la masse totale de l'agent pénétrant, en particulier de 0,01% en poids à 30% en poids, mieux encore de 0,1% en poids à 25% en poids et de la façon la plus préférentielle de 0,5% en poids à 15% en poids.

22. Utilisation selon l'une quelconque des revendications 1 à 21, dans laquelle le milieu portant les médicaments et les véhicules est un tampon biocompatible ayant une valeur de pH allant de 4 à 10, plus fréquemment de 5 à 9 et le plus souvent de 6 à 8.

23. Utilisation selon l'une quelconque des revendications 1 à 22, dans laquelle les additifs sont inclus dans la préparation afin de réduire la sensibilité du système aux contraintes chimiques, biologiques ou ambiantes et comprennent des antioxydants, des antagonistes des actions enzymatiques indésirables, des cryoconservateurs, des microbicides, etc., ou bien des modulateurs de propriétés physiquement importantes du système telles que la viscosité de la formulation, etc.

24. Utilisation selon l'une quelconque des revendications 1 à 23, dans laquelle la dose relative du médicament ou de l'agent à administrer de manière non invasive par le nez au moyen de véhicules hautement adaptables est choisie de manière à être entre 0,1 fois et 500 fois, plus souvent entre 0,5 fois et 250 fois et de façon encore plus préférentielle entre 1 fois et 100 fois différente de la dose correspondante de médicament ou d'agent qu'il faudrait injecter pour obtenir les effets biologiques souhaités.

25. Utilisation selon l'une quelconque des revendications 1 à 24, dans laquelle la dose d'agent pénétrant appliquée est de 0,01 mg à 15 mg par narine, plus souvent encore elle se situe dans la plage de 0,1 mg à 10 mg par narine et de préférence de 0,5 mg à 5 mg par narine.

26. Utilisation selon l'une quelconque des revendications 1 à 25, dans laquelle l'efficacité de l'administration et les effets biologiques de l'agent ou du médicament choisi sont contrôlés en utilisant des volumes d'application différents.

27. Utilisation selon l'une quelconque des revendications 1 à 26, dans laquelle ladite formulation est administrée en utilisant un dispositif de délivrance volumétrique.

28. Utilisation selon l'une quelconque des revendications 1 à 27, dans laquelle différents volumes d'application sont choisis pour contrôler l'efficacité de l'administration et les effets biologiques de l'agent ou du médicament choisi.

29. Utilisation selon l'une quelconque des revendications 1 à 28, dans laquelle les agents pénétrants en suspension sont chargés avec les médicaments ou agents dans un délai de 24 heures avant d'administration de la formulation, de préférence 360 minutes, de façon plus préférentielle 60 minutes et de façon encore plus préférentielle 30 minutes avant l'administration de la formulation ainsi obtenue dans le nez.

30. Utilisation selon l'une quelconque des revendications 1 à 29, dans laquelle le dispositif de délivrance est chargé sur le lieu de traitement.

31. Utilisation selon l'une quelconque des revendications 1 à 30, dans laquelle le dispositif est chargé séparément avec des agents pénétrants et des molécules, en particulier des agents biologiques, à associer à ceux-ci.

32. Utilisation selon l'une quelconque des revendications 1 à 31, dans laquelle le principe actif pharmaceutique est destiné à l'administration au système nerveux.

33. Utilisation selon la revendication 32, dans laquelle le système nerveux est le cerveau.

34. Utilisation selon l'une quelconque des revendications 1 à 33, dans laquelle ladite composition pharmaceutique est un vaccin.

35. Utilisation selon la revendication 34, dans laquelle le vaccin comprend en plus un extrait d'agent pathogène ou un composé provenant d'un agent pathogène ou un fragment ou un dérivé de celui-ci.

36. Utilisation selon la revendication 35, dans laquelle ledit extrait d'agent pathogène ou composé est choisi parmi le virus de l'hépatite, le virus de l'immunodéficience (humaine), les herpèsvirus, les virus de la variole (varicelle), de la grippe, de la rougeole, des oreillons ou de la poliomyélite, le cytomégalovirus, le rhinovirus, etc., ou des champignons qui prospèrent à l'intérieur de cellules hôtes, un parasite, comprenant des parasites des animaux tels que les protozoaires et les helminthes, et des ectoparasites tels que les tiques et les acariens,

ou les espèces *Brucella*, comprenant l'agent responsable du choléra, les espèces *Haemophilus*, ainsi que des agents pathogènes qui déclenchent la fièvre paratyphoïde, la peste, la rage, le tétanos ou la rubéole.

37. Utilisation selon l'une quelconque des revendications 34 à 36, dans laquelle ledit vaccin comprend en plus un adjuvant.

38. Utilisation selon la revendication 37, dans laquelle ledit adjuvant est un lipopolysaccharide tel que le lipide A ou un dérivé ou une modification de celui-ci, par exemple le lipide A monophosphorylé, ou son analogue, par exemple un dérivé gras de saccharose, un facteur cordonal (tréhalose dimycolate), un dipeptide muramylique ou un autre (poly) saccharide ou (poly)peptide identique ou ressemblant à une partie immunologiquement active d'une membrane d'un micro-organisme ; un extrait d'un micro-organisme comprenant des exotoxines et endotoxines bactériennes, de préférence la toxine du choléra ou la toxine thermolabile de *E. coli*, un dérivé de chaîne A, un composant ayant une activité de ribosylation de l'ADP, un peptidoglycane, une toxine de *Clostridia,* une halotoxine LT, un dérivé protéinique purifié de *M. tuberculosis,* le LT-R192G, la protéine I liant la fibronectine de *Streptococcus pyogenes* ou une protéine de la membrane externe de *Neisseria meningitidis* du groupe B (GBOMP).

39. Utilisation selon l'une quelconque des revendications 34 à 38, dans laquelle ledit vaccin comprend un mélange de MPL et d'IL-12 ou de GM-CSF et d'IL-4.

40. Utilisation selon l'une quelconque des revendications 34 à 39, dans laquelle, dans ledit vaccin, la dose relative d'immunogène/antigène à administrer de manière non invasive par le nez au moyen de véhicules hautement adaptables est choisie de manière à être entre 0,01 fois et 100 fois, plus souvent entre 0,05 fois et 75 fois et de façon encore plus préférentielle entre 0,1 fois et 50 fois différente de la dose correspondante d'immunogène/antigène qu'il faudrait injecter pour obtenir l'effet biologique souhaité.

41. Utilisation selon l'une quelconque des revendications 37 à 40, dans laquelle, dans ledit vaccin, la concentration de l'adjuvant administré par voie transnasale se situe dans la plage de 10 fois moins jusqu'à 1000 fois plus que celle utilisée avec les formulations correspondantes employant un antigène similaire et injectées par voie sous-cutanée, la concentration de l'immunoadjuvant administré par voie transnasale différant plus souvent de la concentration de l'immunoadjuvant injecté par un facteur allant de 0,5 à 100 ou mieux d'un facteur allant de 1 à 50 et mieux encore de 2 à 25.

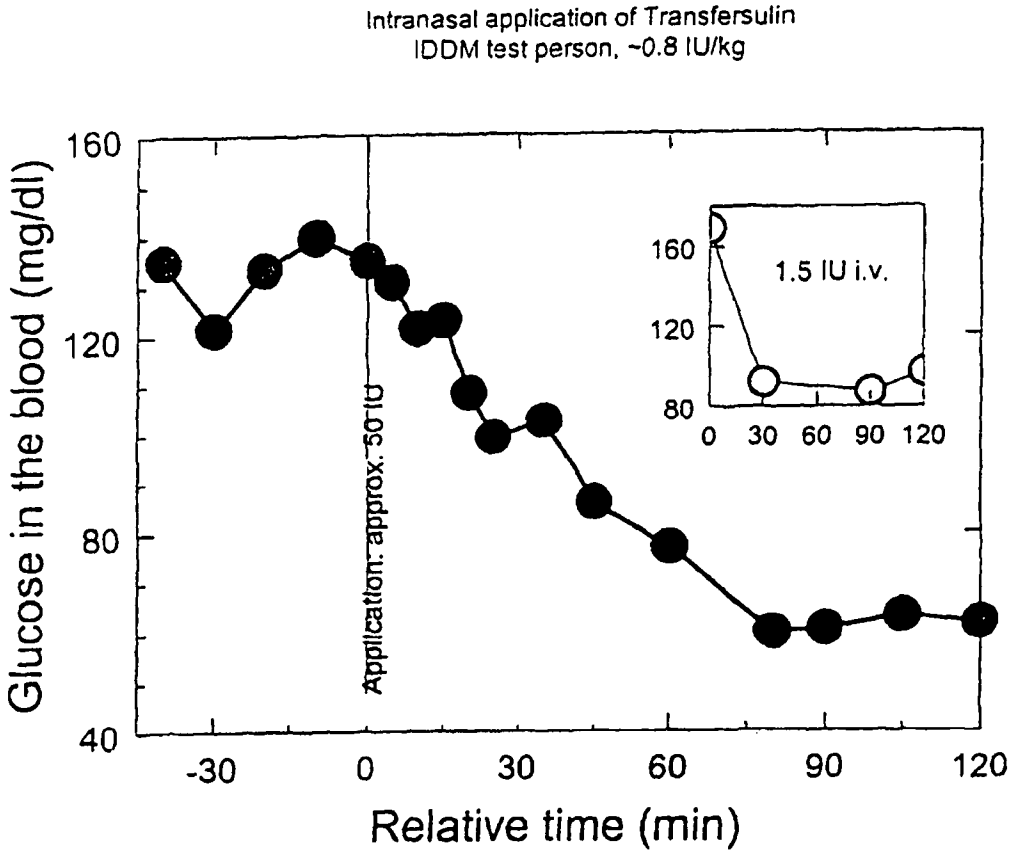
Intranasal application of Transfersulin
IDDM test person, ~0.8 IU/kg
Glucose in the blood (mg/dl)
160
120
80
40
-30
0
30
60
90
120
Relative time (min)
Application: approx. 50 IU
1.5 IU i.v.
160
120
80
0
30
60
90
120

Figure 1

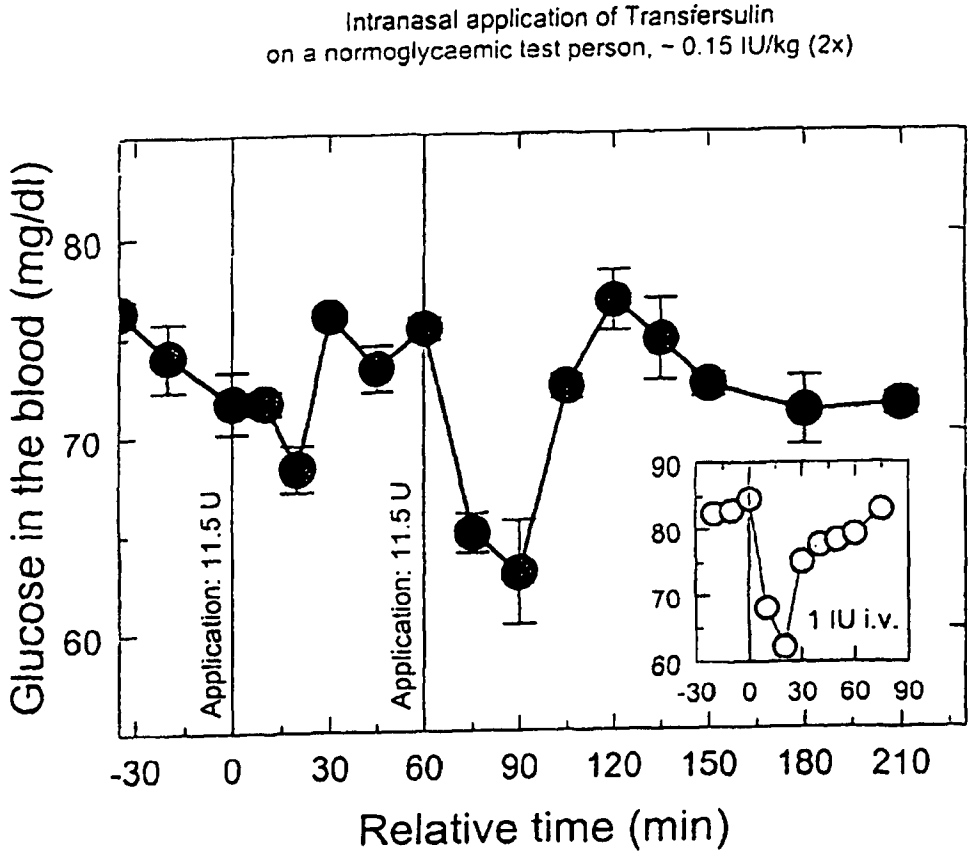
Intranasal application of Transfersulin
on a normoglycaemic test person, ~ 0.15 IU/kg (2x)
Glucose in the blood (mg/dl)
80
70
60
Application: 11.5 U
Application: 11.5 U
-30
0
30
60
90
120
150
180
210
Relative time (min)
90
80
70
60
1 IU i.v.
-30
0
30
60
90

Figure 2

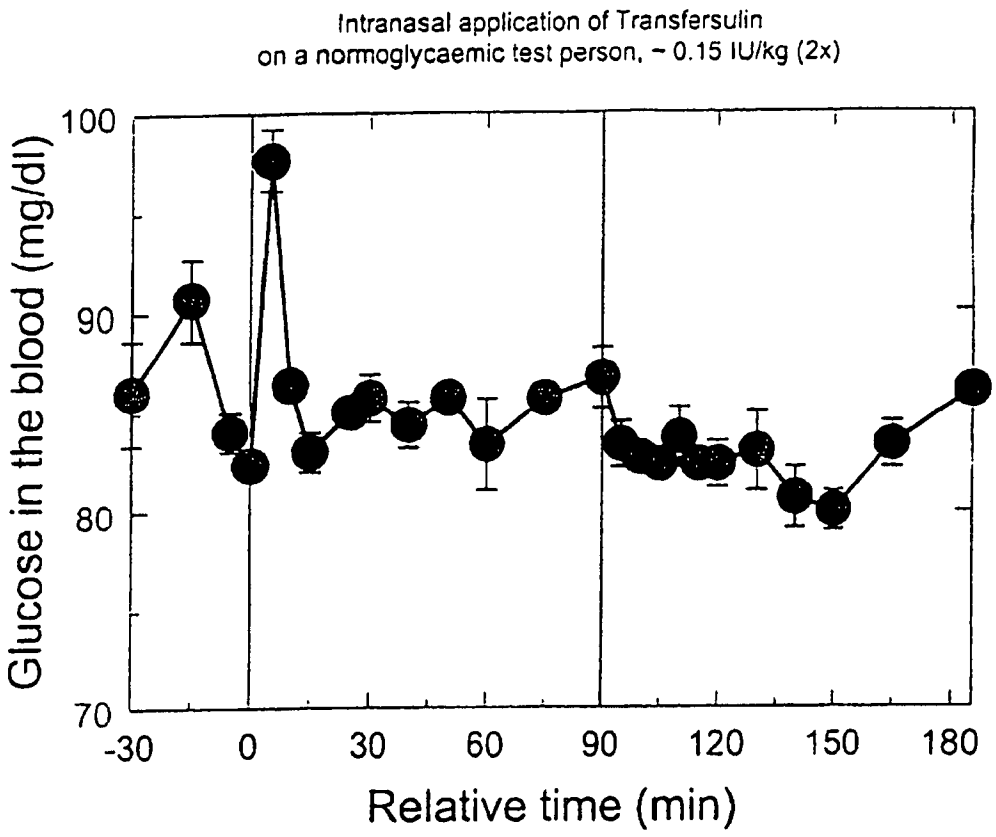
Intranasal application of Transfersulin
on a normoglycaemic test person, ~ 0.15 IU/kg (2x)
Glucose in the blood (mg/dl)
100
90
80
70
-30
0
30
60
90
120
150
180
Relative time (min)

Figure 3a

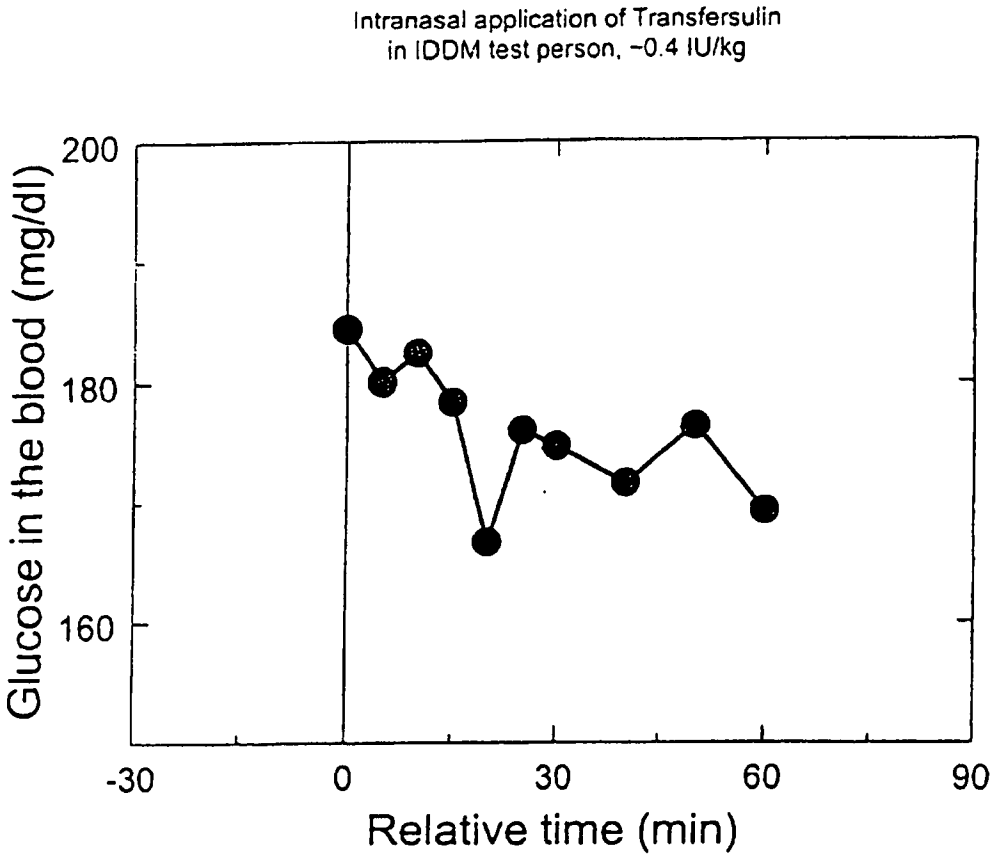
Intranasal application of Transfersulin
in IDDM test person, −0.4 IU/kg
Glucose in the blood (mg/dl)
200
180
160
-30
0
30
60
90
Relative time (min)

Figure 3b

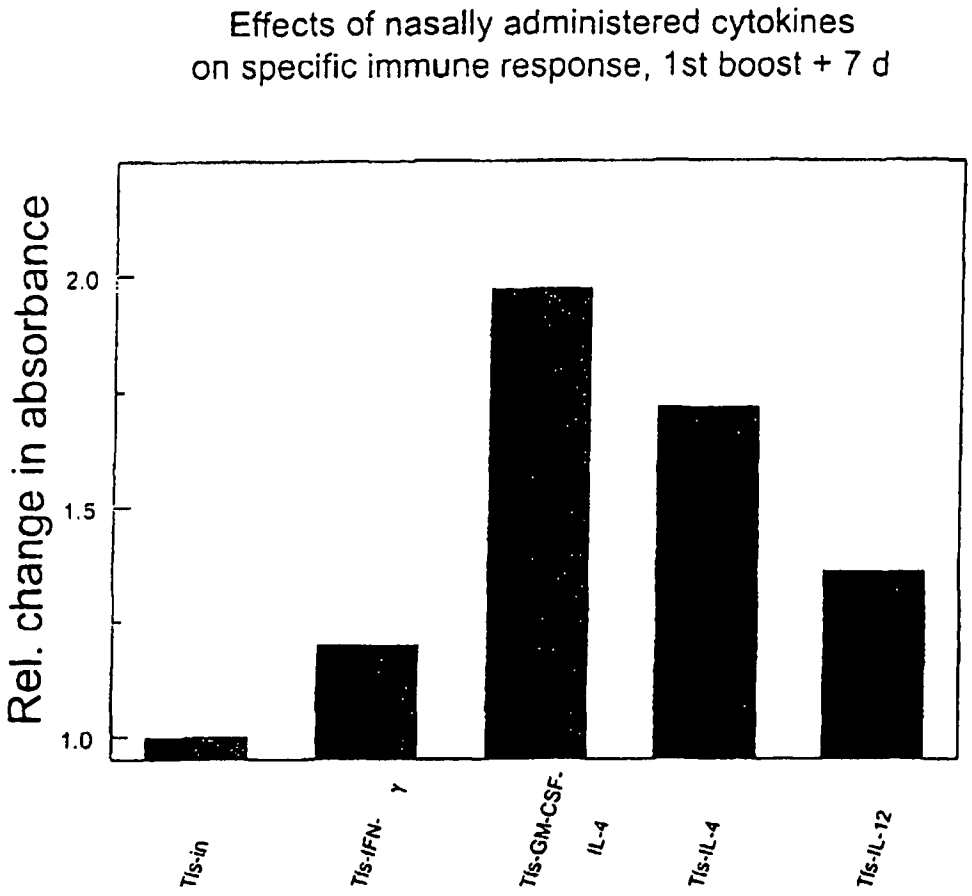
Effects of nasally administered cytokines
on specific immune response, 1st boost + 7 d
Rel. change in absorbance
2.0
1.5
1.0
Tfs-in
Tfs-IFN-γ
Tfs-GM-CSF-IL-4
Tfs-IL-4
Tfs-IL-12
Tfs: SPC:Tw-80 (1:1)
n=6

Figure 4

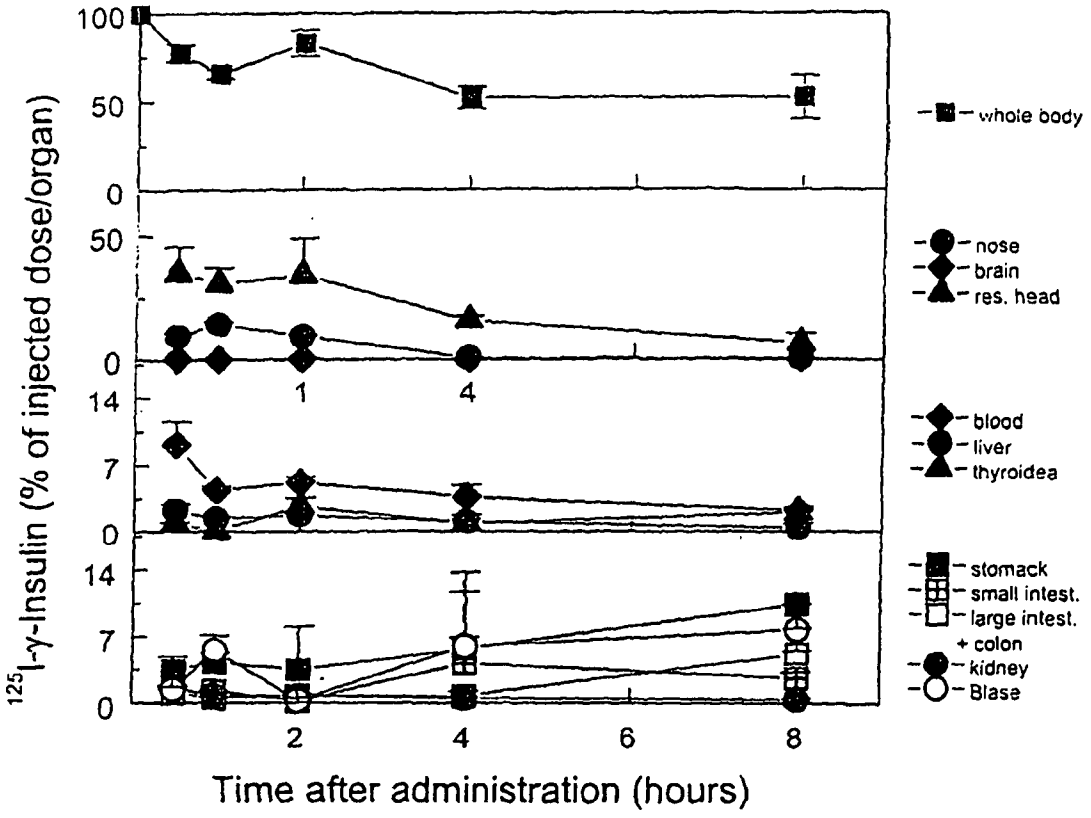
100
50
0
50
0
14
7
0
14
7
0
1
4
2
4
6
8
^{125}I-γ-Insulin (% of injected dose/organ)
Time after administration (hours)
whole body
nose
brain
res. head
blood
liver
thyroidea
stomack
small intest.
large intest.
+ colon
kidney
Blase

Figure 5

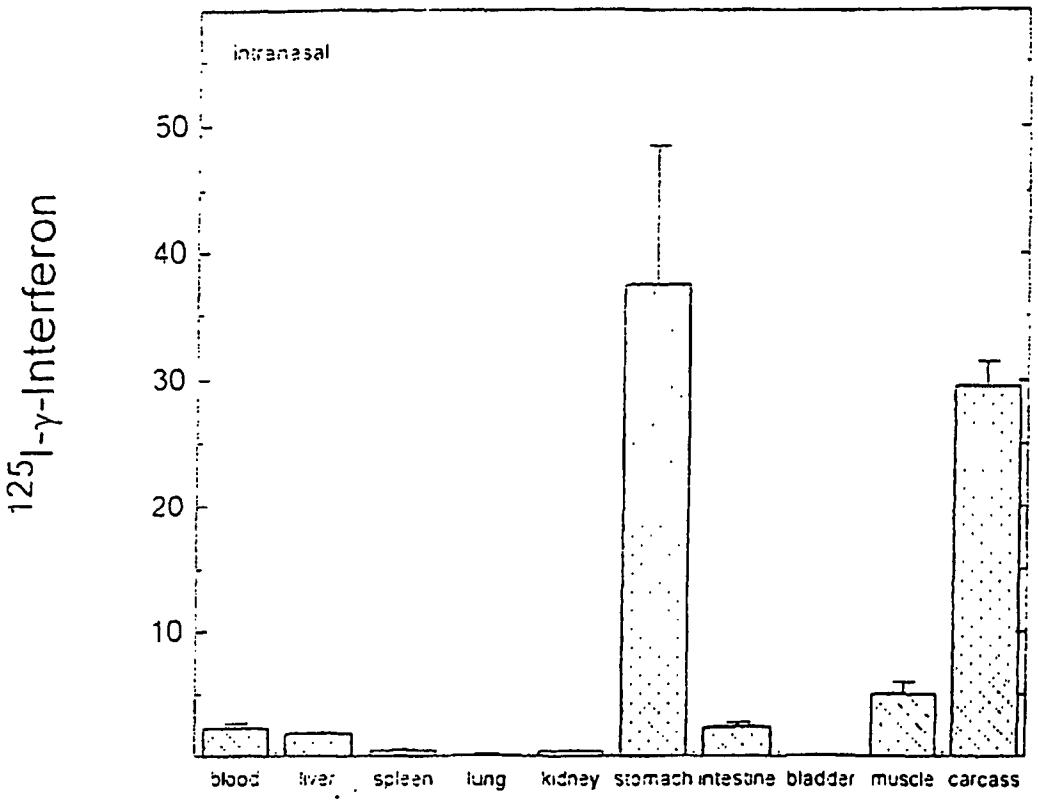
intranasal
^{125}I-γ-Interferon
50
40
30
20
10
blood
liver
spleen
lung
kidney
stomach
intestine
bladder
muscle
carcass

Figure 6

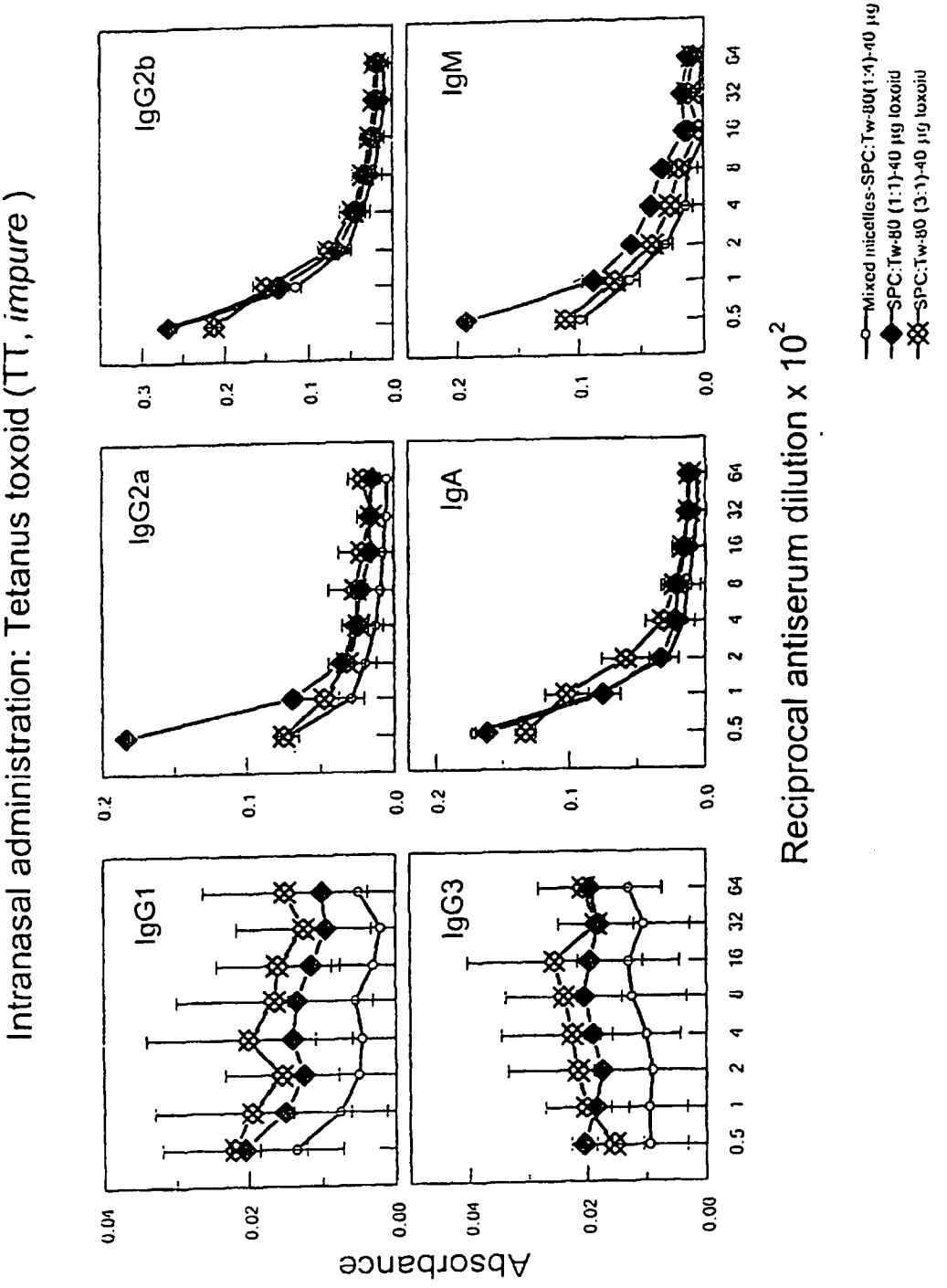
Intranasal administration: Tetanus toxoid (TT, impure)
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Absorbance
Reciprocal antiserum dilution x 10²
0.5 1 2 4 8 16 32 64
Mixed micelles-SPC:Tw-80(1:1)-40 µg toxoid
SPC:Tw-80 (1:1)-40 µg toxoid
SPC:Tw-80 (3:1)-40 µg toxoid

Figure 7a

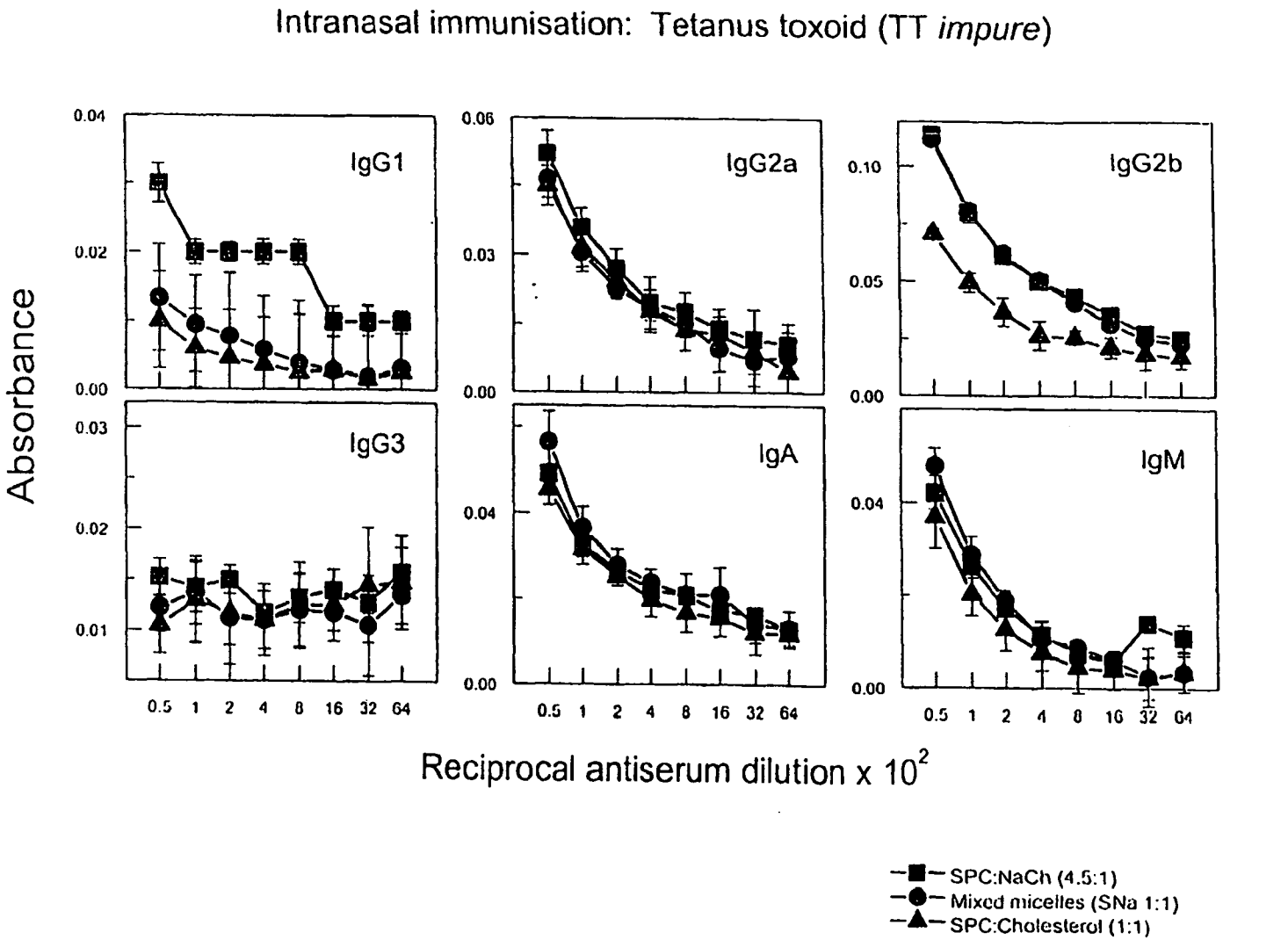
Intranasal immunisation: Tetanus toxoid (TT *impure*)
Absorbance
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
0.5 1 2 4 8 16 32 64
Reciprocal antiserum dilution x 10^2
SPC:NaCh (4.5:1)
Mixed micelles (SNa 1:1)
SPC:Cholesterol (1:1)
Antigen dose in all the groups : 40 µg per mouse

Figure 7b

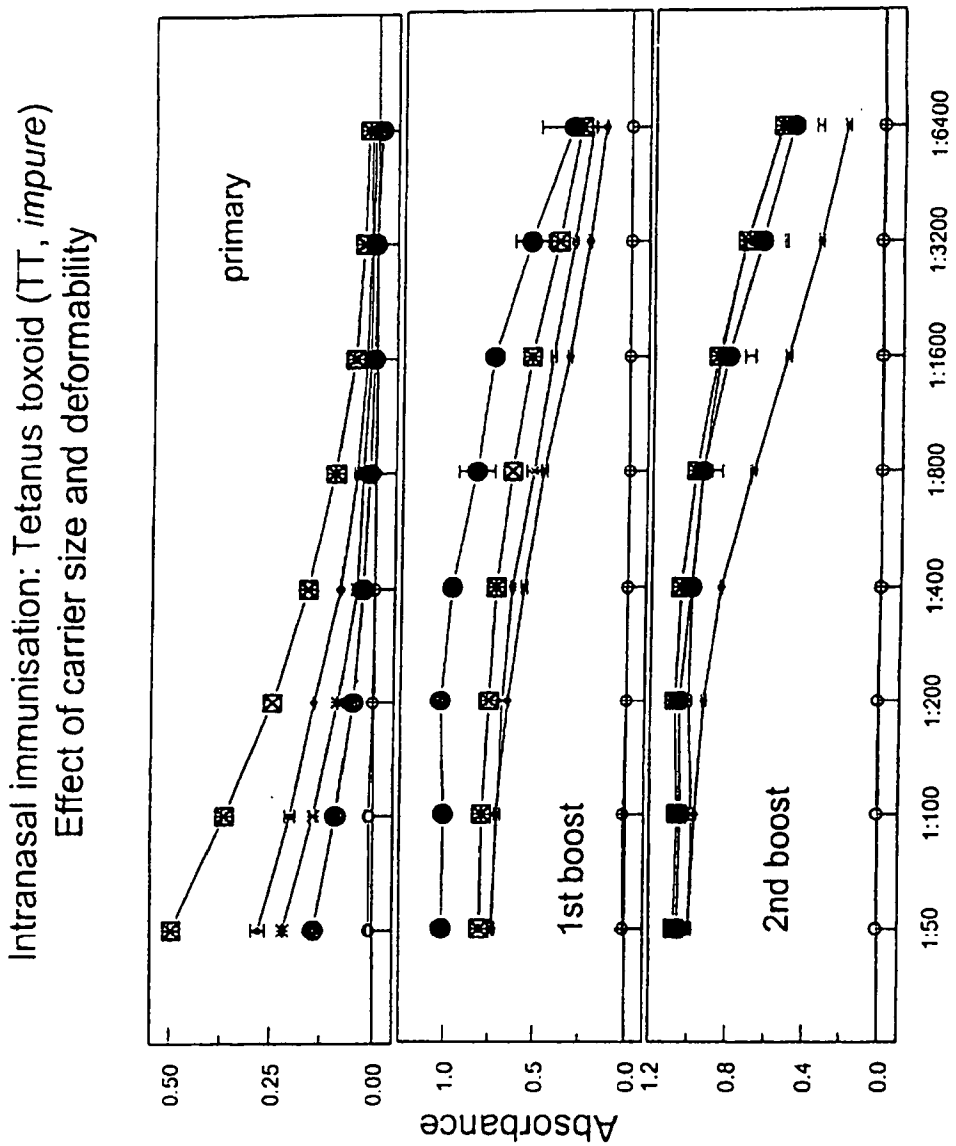
Intranasal immunisation: Tetanus toxoid (TT, *impure*)
Effect of carrier size and deformability
primary
1st boost
2nd boost
Absorbance
0.50
0.25
0.00
1.0
0.5
0.0
1.2
0.8
0.4
0.0
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
Antiserum dilution
unsplit TTs
40 µg TT in TTs (SPC:NaCh 3.75:1)
40 µg TT in Lip (SPC:Cholesterol 1:1)
40 µg TT in MM(SPC:Tw-80 1:8)
40 µg TT in MM(SPC:NaCh 1:4)

Figure 7c

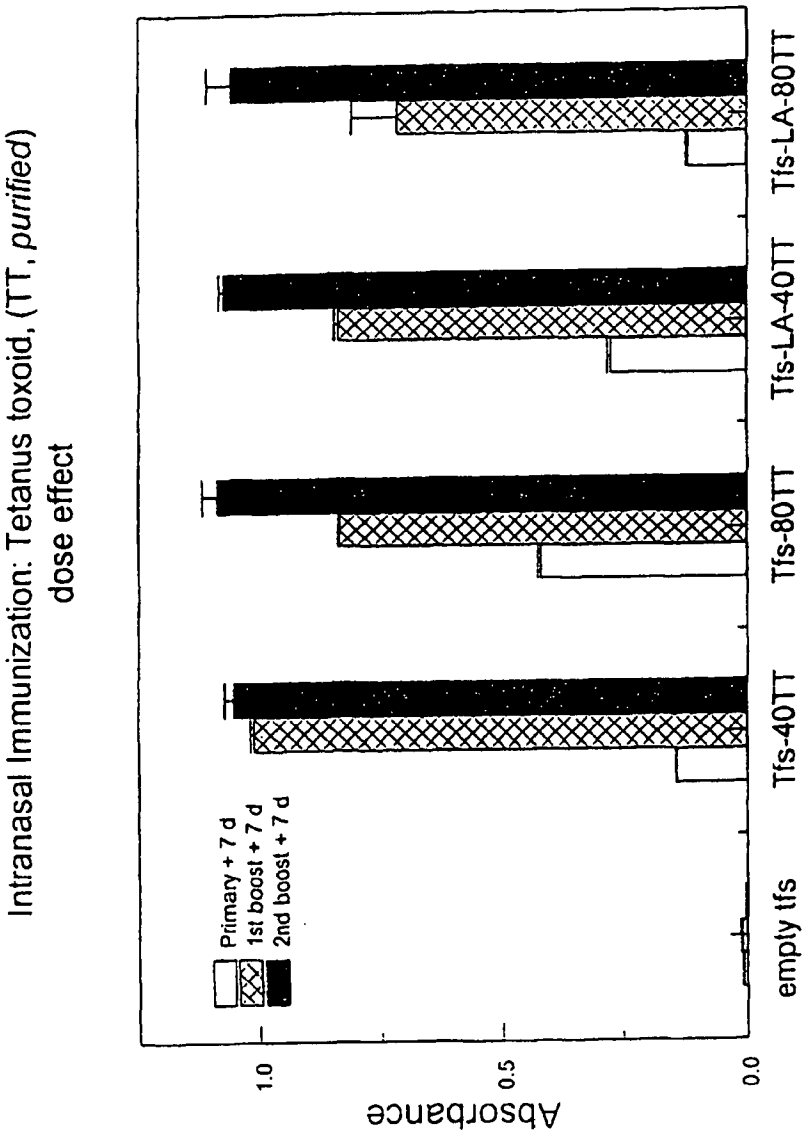
Intranasal Immunization: Tetanus toxoid, (TT, *purified*)
dose effect
Absorbance
1.0
0.5
0.0
Primary + 7 d
1st boost + 7 d
2nd boost + 7 d
empty tfs
Tfs-40TT
Tfs-80TT
Tfs-LA-40TT
Tfs-LA-80TT

Figure 8a

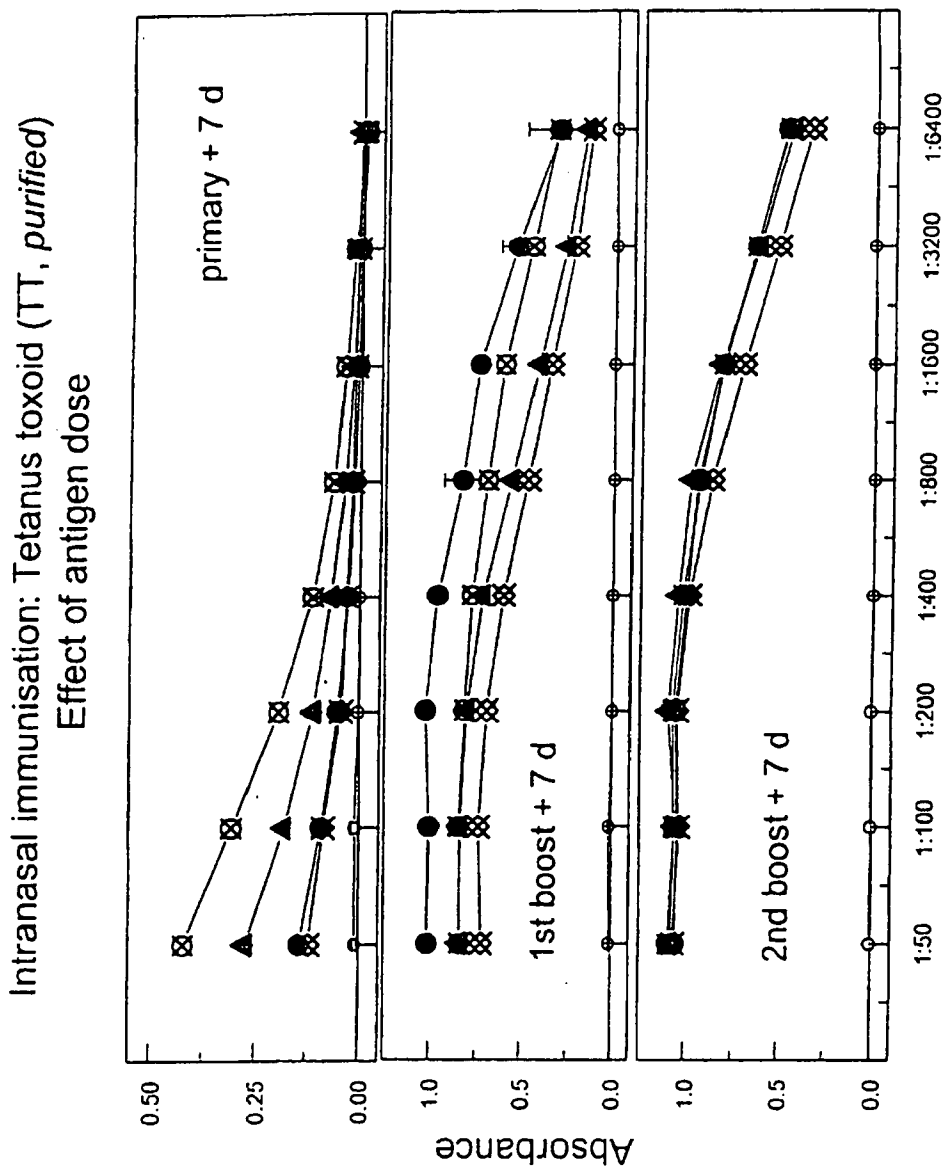
Intranasal immunisation: Tetanus toxoid (TT, *purified*)
Effect of antigen dose
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Absorbance
0.50
0.25
0.00
1.0
0.5
0.0
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400
Antiserum dilution
empty Tls
40 µg TT in Tls (SPC:NaCh 3.75:1)
40 µg TT in Tls+LA (SPC:NaCh 3.75:1)
80 µg TT in Tls (SPC:NaCh 3.75:1)
80 µg TT in Tls+LA (SPC:NaCh3.75:1)

Figure 8b

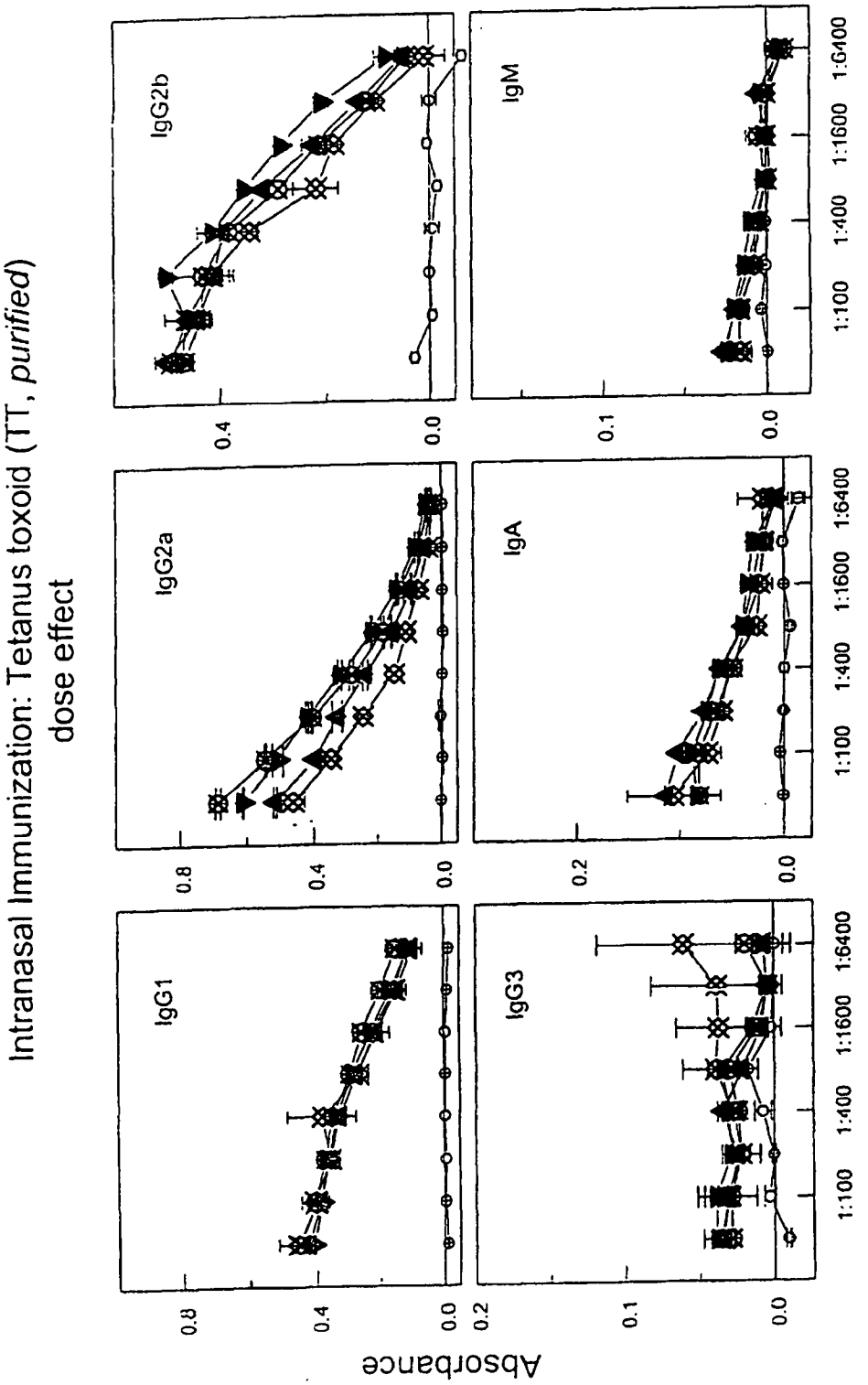
Intranasal Immunization: Tetanus toxoid (TT, purified)
dose effect
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Absorbance
Antiserum dilution
1:100
1:400
1:1600
1:6400
empty Tfs
40 µg TT in Tfs+LA(SPC:NaCh 3.75:1)
40 µg TT in Tfs+LA+IL-12(SPC:NaCh 3.75:1)
90 µg TT in Tfs(SPC:NaCh 3.75:1)
80 µg TT in Tfs+LA(SPC:NaCh 3.75:1)

Figure 8c

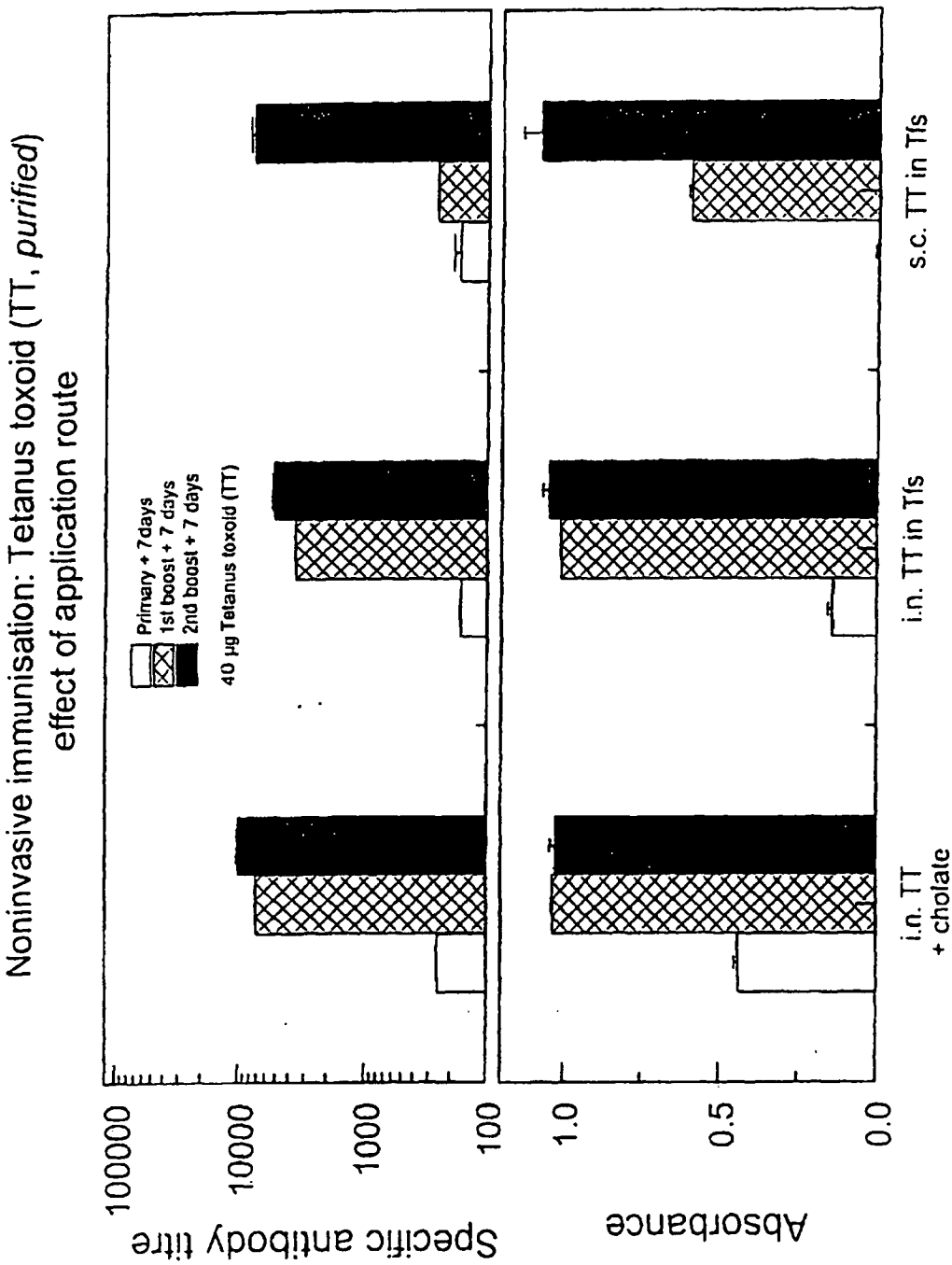
Noninvasive immunisation: Tetanus toxoid (TT, *purified*)
effect of application route
Primary + 7 days
1st boost + 7 days
2nd boost + 7 days
40 µg Tetanus toxoid (TT)
Specific antibody titre
100000
10000
1000
100
Absorbance
1.0
0.5
0.0
i.n. TT
+ cholate
i.n. TT in Tfs
s.c. TT in Tfs

Figure 9a

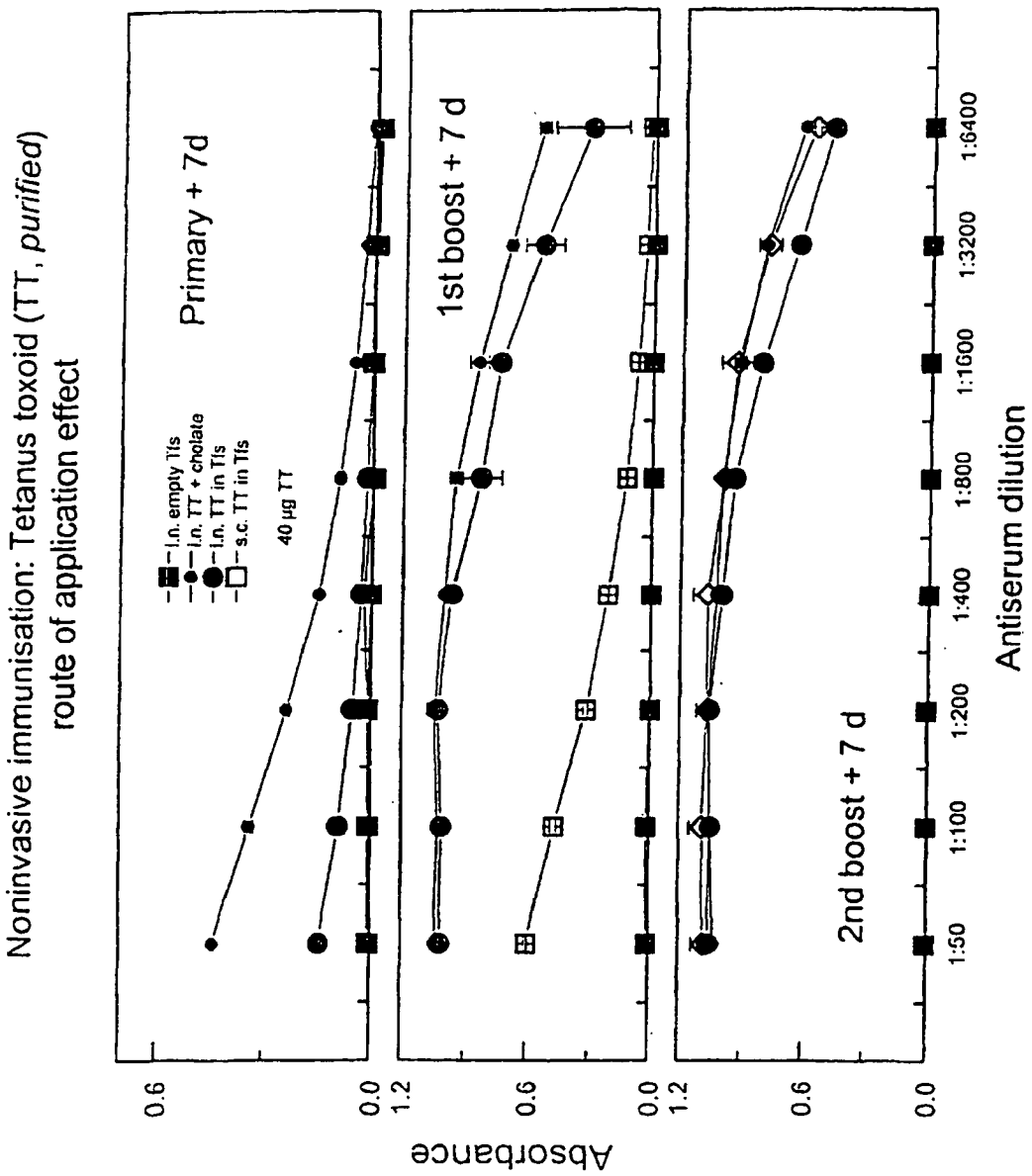
Noninvasive immunisation: Tetanus toxoid (TT, *purified*)
route of application effect
Primary + 7d
1st boost + 7 d
2nd boost + 7 d
i.n. empty TIs
i.n. TT + cholate
i.n. TT in TIs
s.c. TT in TIs
40 µg TT
Absorbance
0.0
0.6
1.2
Antiserum dilution
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:6400

Figure 9b

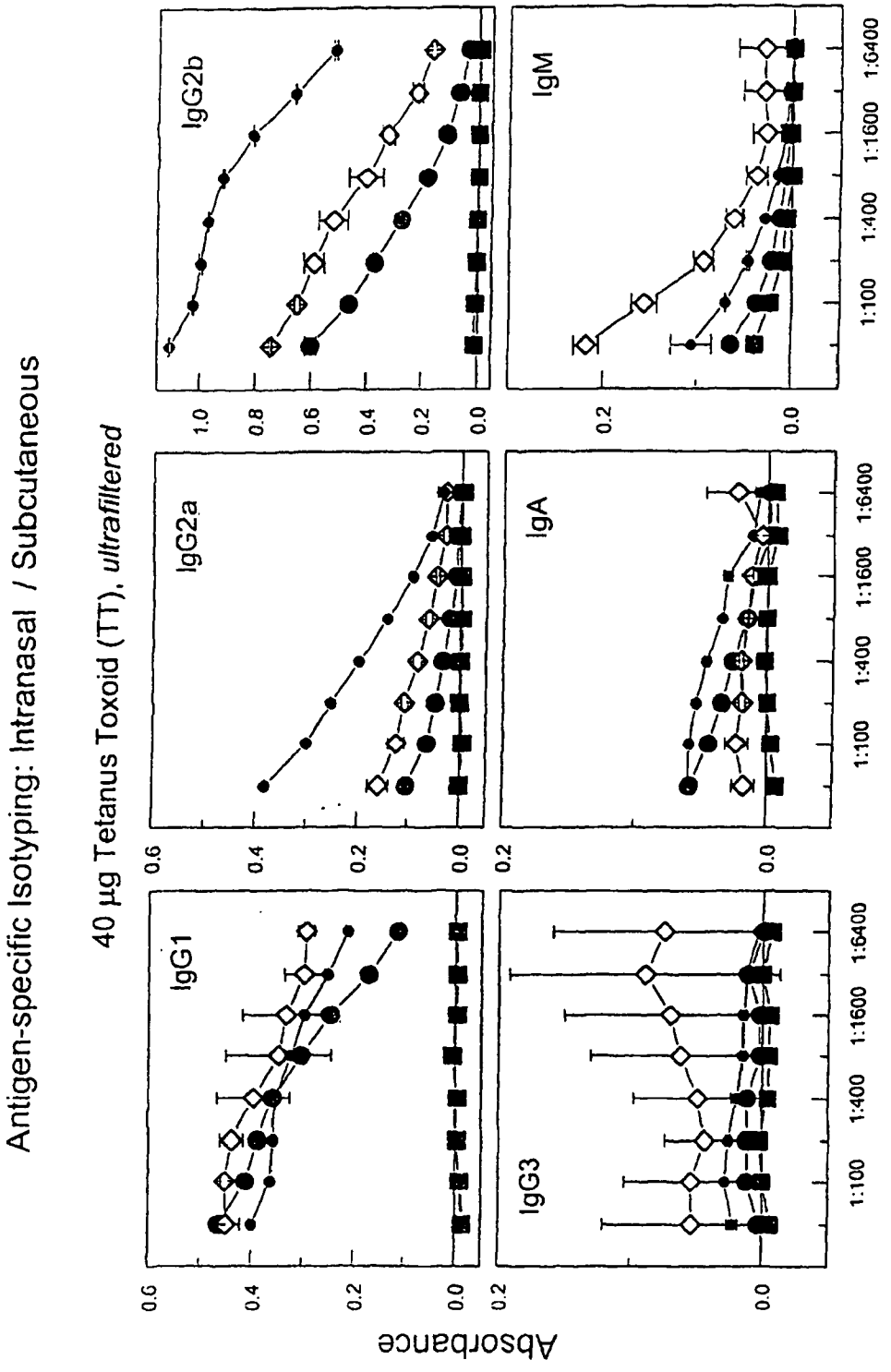
Antigen-specific Isotyping: Intranasal / Subcutaneous
40 µg Tetanus Toxoid (TT), ultrafiltered
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Absorbance
Antiserum dilution
1:100
1:400
1:1600
1:6400
i.n. empty Tfs
i.n. TT + cholate
i.n. TT in Tfs
s.c. TT in Tfs

Figure 9c

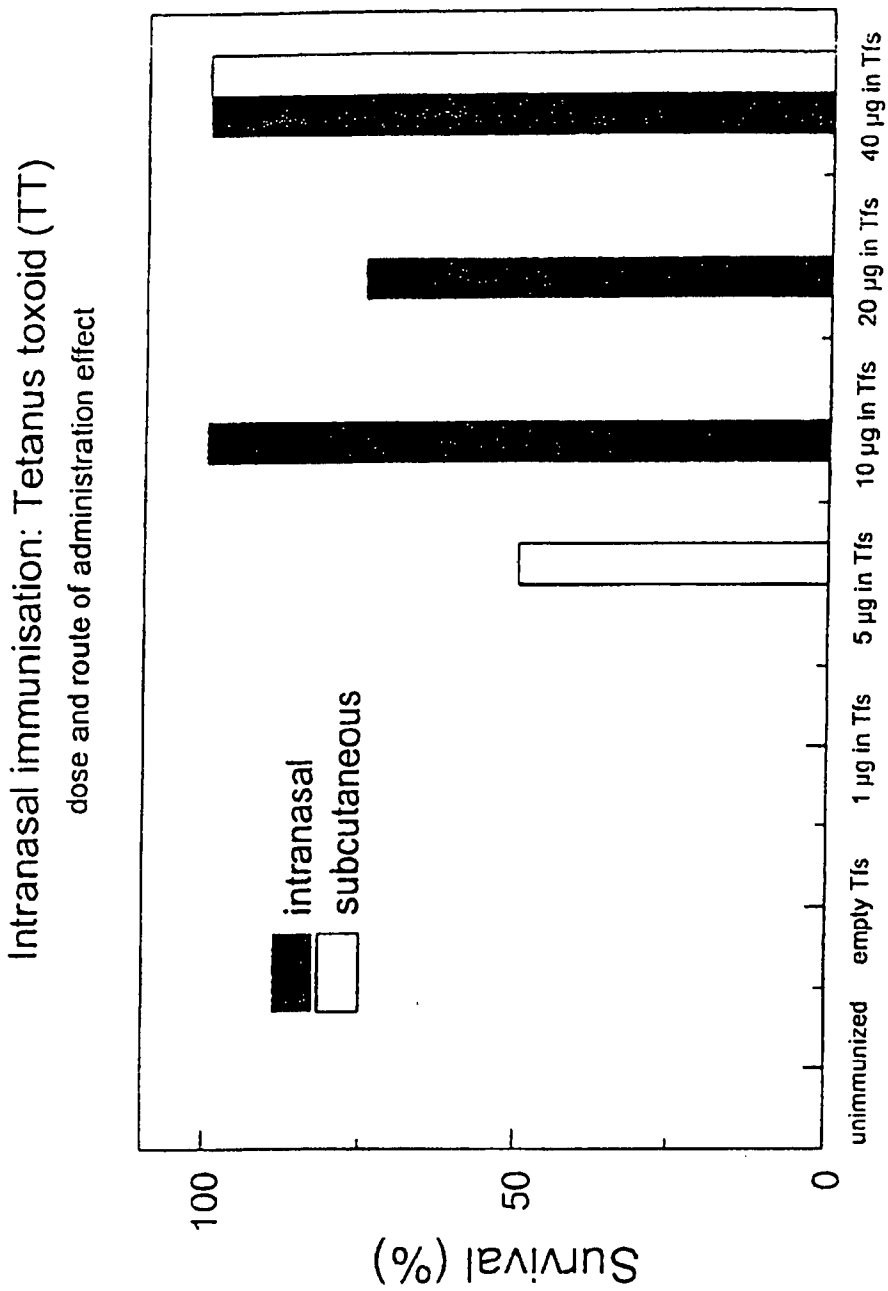
Intranasal immunisation: Tetanus toxoid (TT)
dose and route of administration effect
intranasal
subcutaneous
Survival (%)
100
50
0
unimmunized
emply TFs
1 µg in TFs
5 µg in TFs
10 µg in TFs
20 µg in TFs
40 µg in TFs

Figure 10a

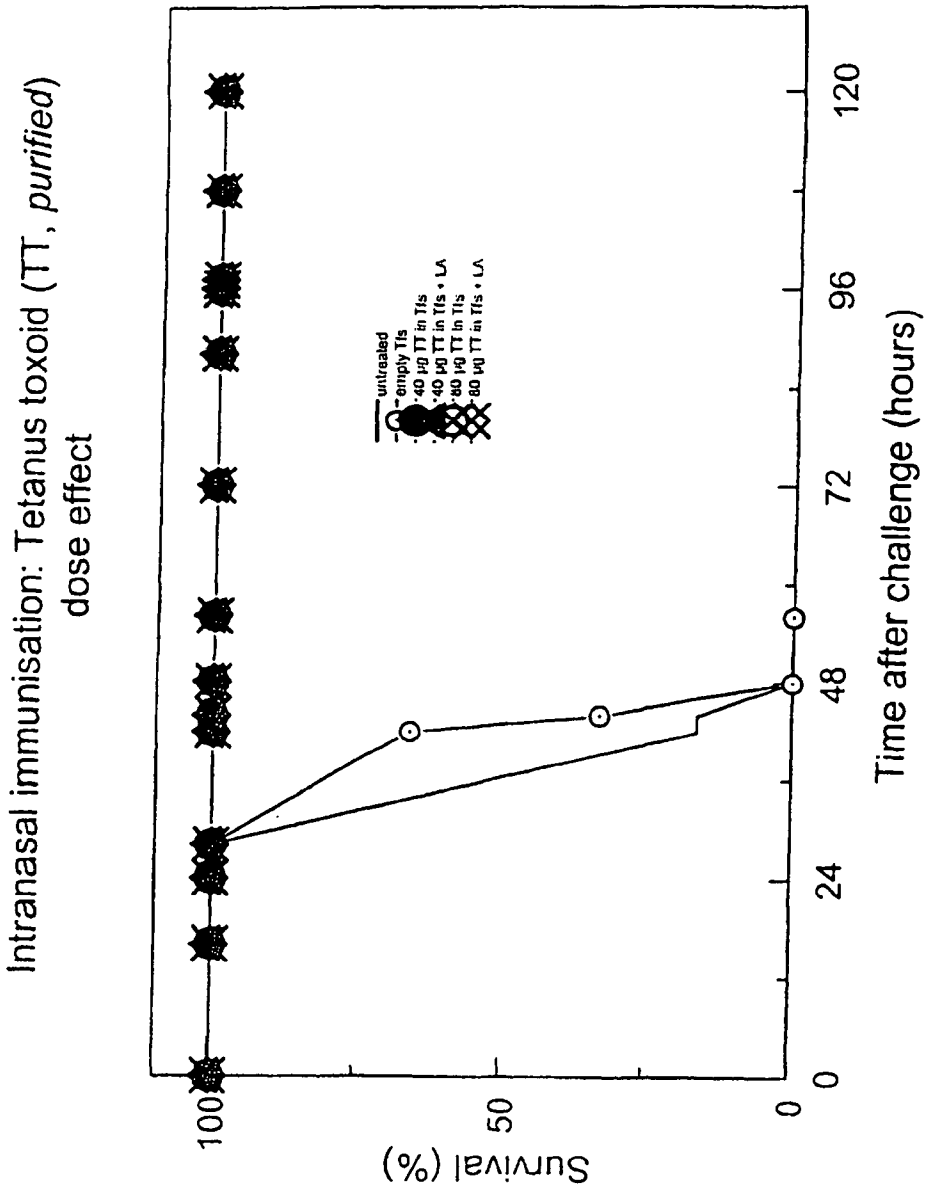
Intranasal immunisation: Tetanus toxoid (TT, *purified*)
dose effect
Survival (%)
100
50
0
0
24
48
72
96
120
Time after challenge (hours)
untreated
empty Tfs
40 µg TT in Tfs
40 µg TT in Tfs + LA
80 µg TT in Tfs
80 µg TT in Tfs + LA

Figure 10b

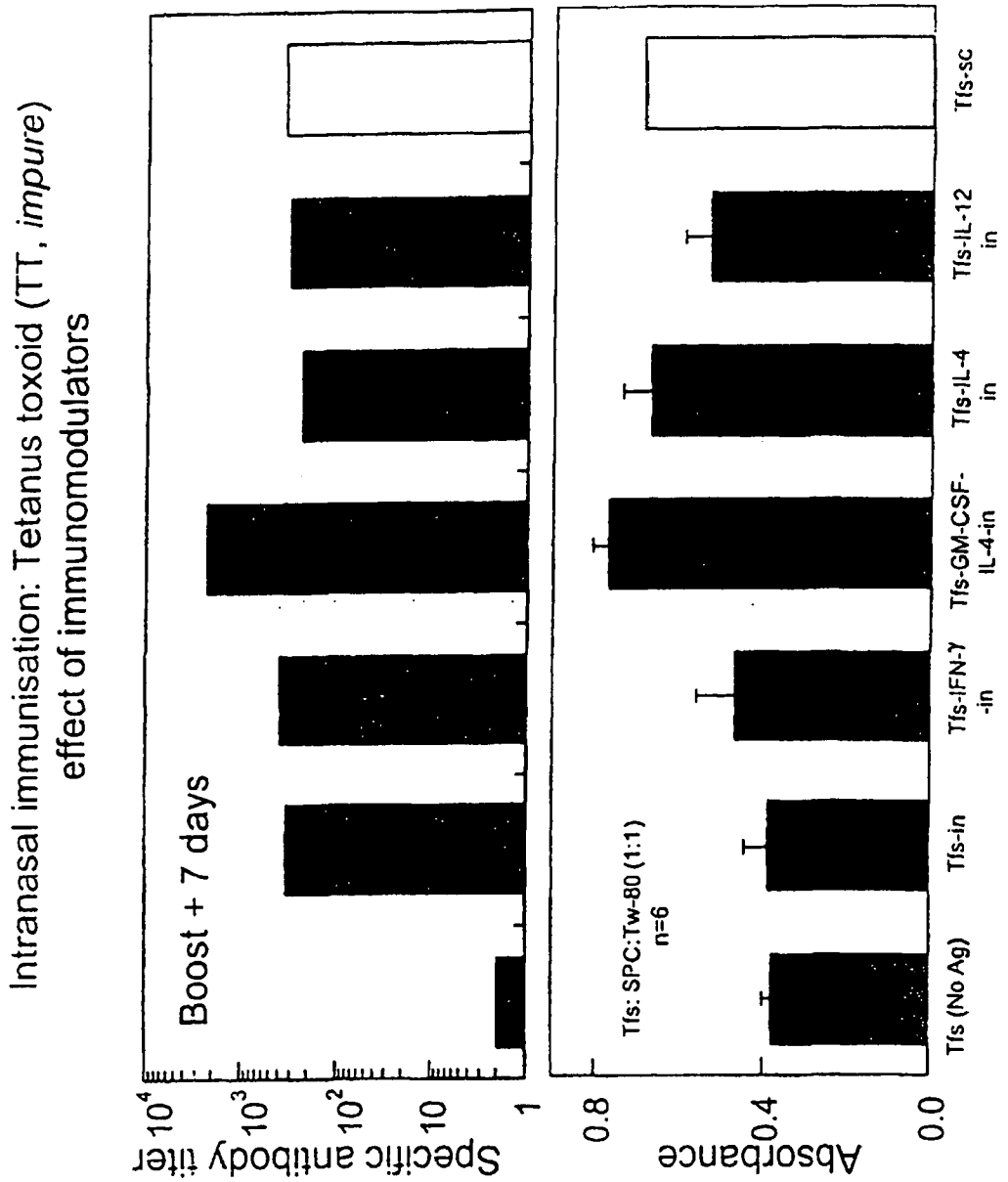
Intranasal immunisation: Tetanus toxoid (TT, *impure*)
effect of immunomodulators
Boost + 7 days
Specific antibody titer
10^4
10^3
10^2
10
1
Absorbance
0.8
0.4
0.0
Tfs: SPC:Tw-80 (1:1)
n=6
Tfs (No Ag)
Tfs-in
Tfs-IFN-γ -in
Tfs-GM-CSF-IL-4-in
Tfs-IL-4 in
Tfs-IL-12 in
Tfs-sc

Figure 11a

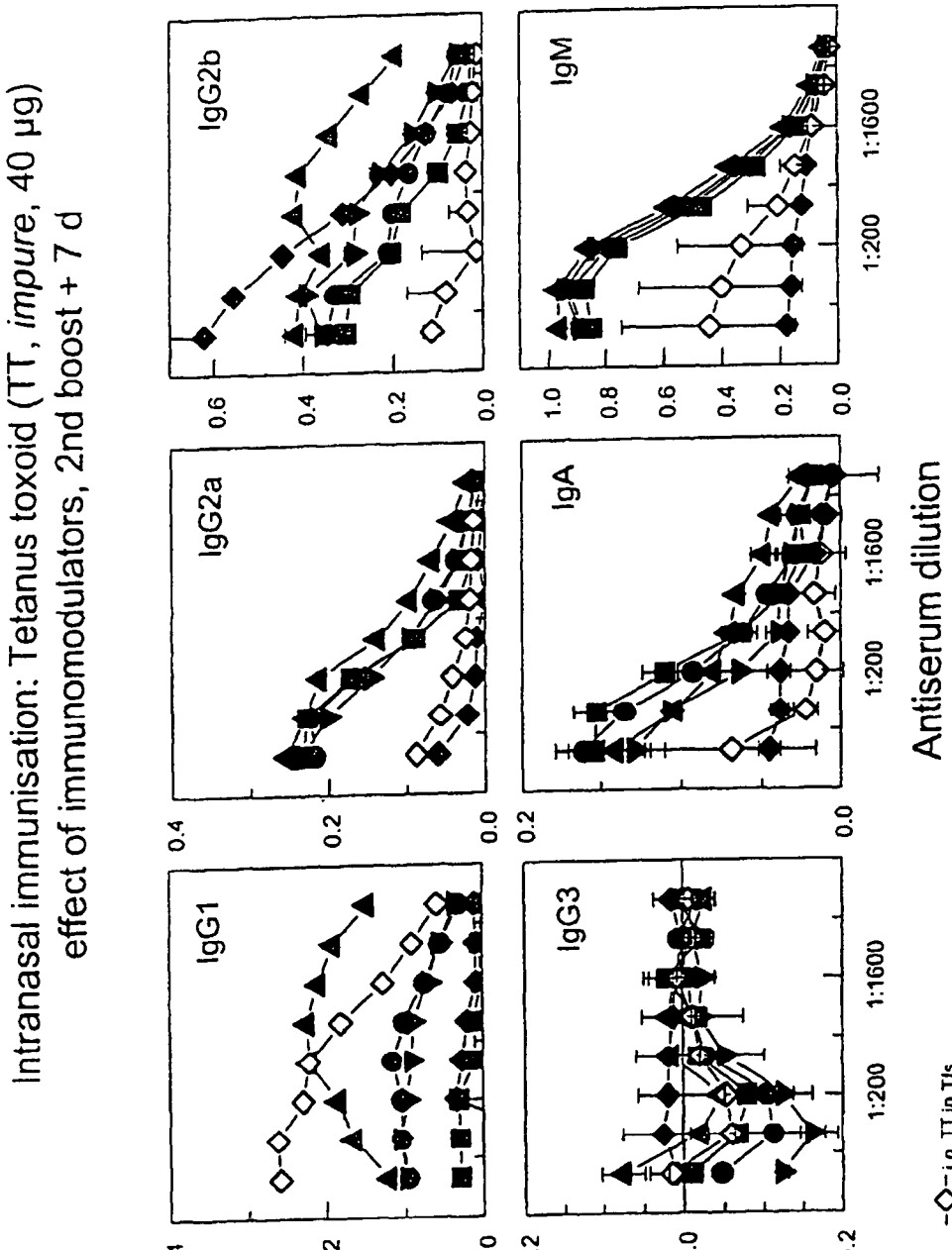
Intranasal immunisation: Tetanus toxoid (TT, *impure*, 40 µg)
effect of immunomodulators, 2nd boost + 7 d
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Absorbance
Antiserum dilution
1:200
1:1600
i.n. TT in TIs
i.n. TT in TIs + IFN-γ
i.n. TT in TIs + GM-CSF + IL-4
i.n. TT in TIs + IL-4
i.n. TT in TIs + IL-12
s.c. TT in TIs

Figure 11b

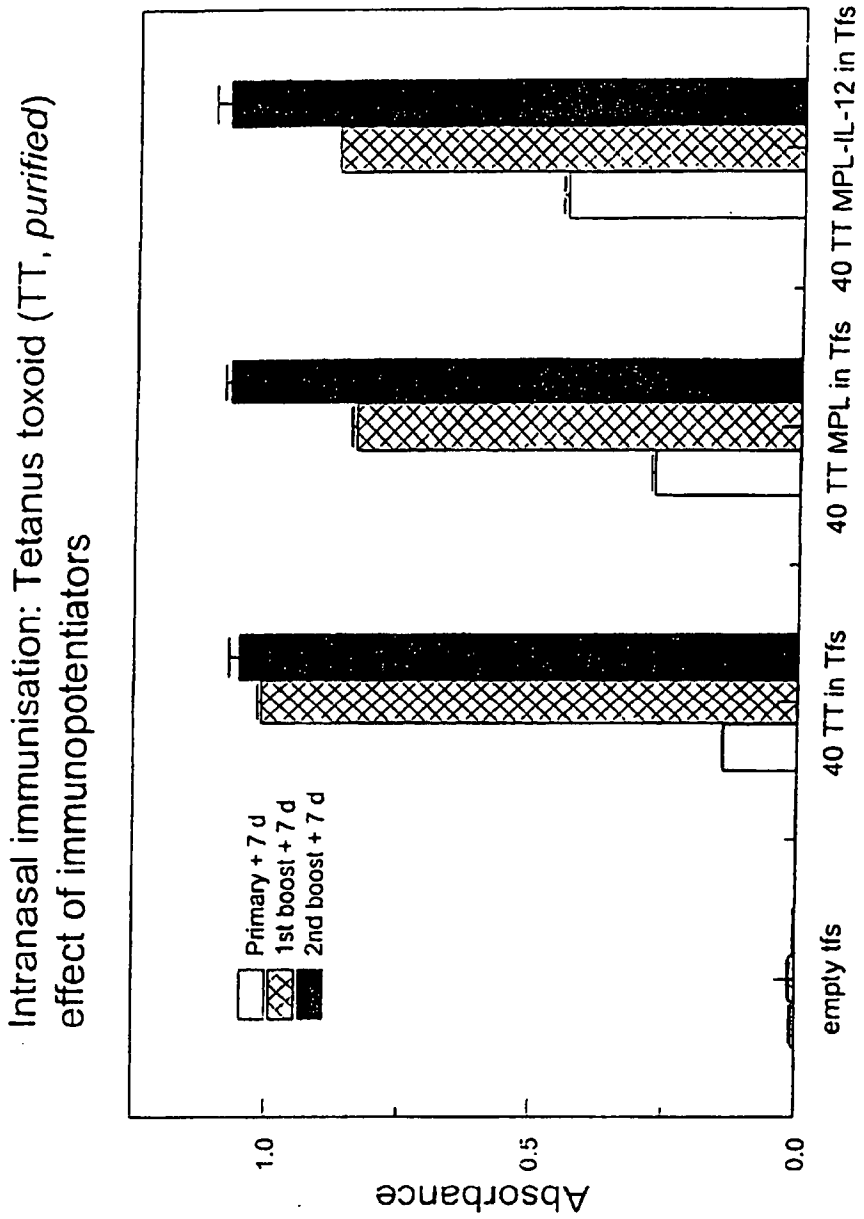
Intranasal immunisation: Tetanus toxoid (TT, *purified*)
effect of immunopotentiators
Absorbance
1.0
0.5
0.0
Primary + 7 d
1st boost + 7 d
2nd boost + 7 d
empty tfs
40 TT in Tfs
40 TT MPL in Tfs
40 TT MPL-IL-12 in Tfs

Figure 12

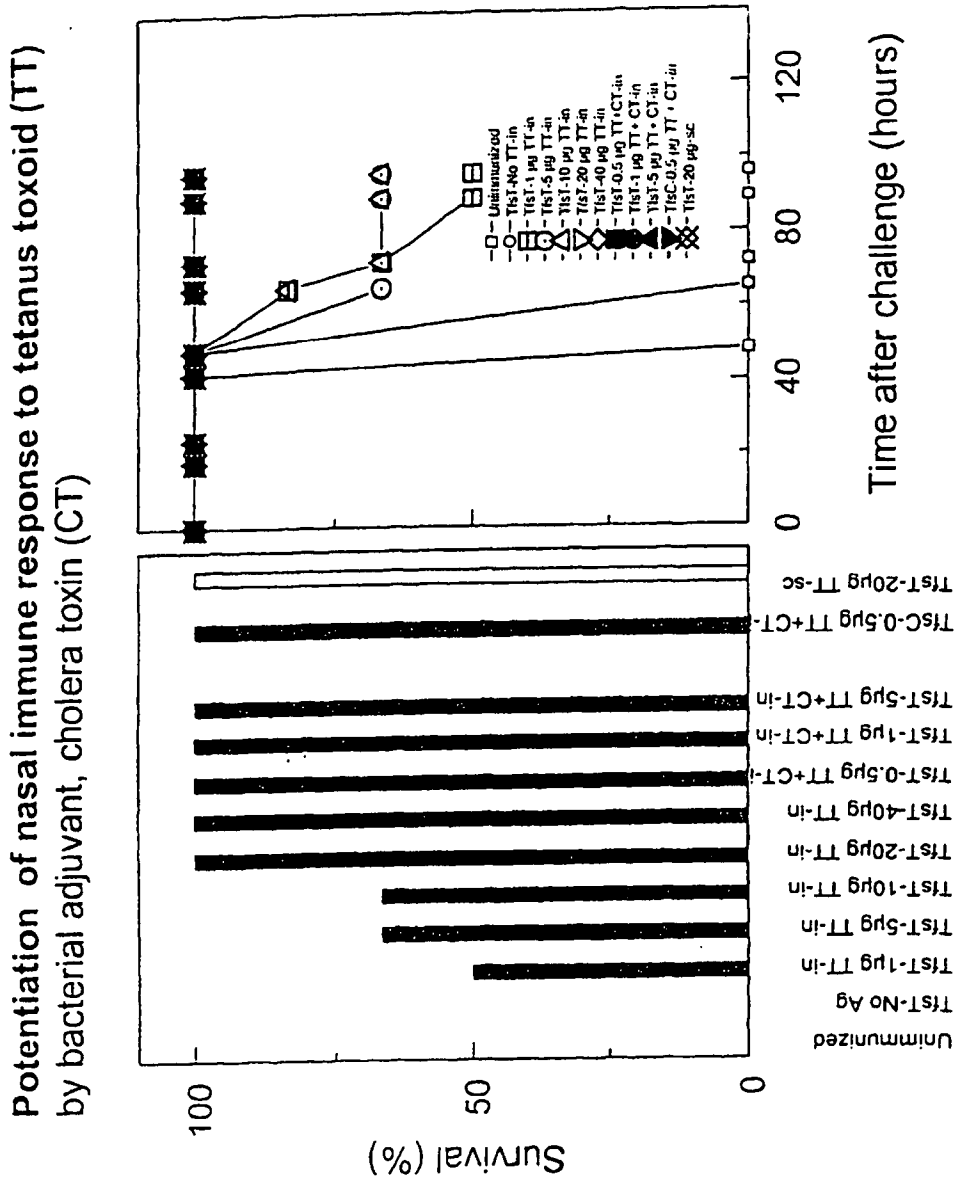
Potentiation of nasal immune response to tetanus toxoid (TT)
by bacterial adjuvant, cholera toxin (CT)
Survival (%)
100
50
0
Unimmunized
TtsT-No Ag
TtsT-1µg TT-in
TtsT-5µg TT-in
TtsT-10µg TT-in
TtsT-20µg TT-in
TtsT-40µg TT-in
TtsT-0.5µg TT+CT-in
TtsT-1µg TT+CT-in
TtsT-5µg TT+CT-in
TtsC-0.5µg TT+CT-in
TtsT-20µg TT-sc
0
40
80
120
Time after challenge (hours)
Unimmunized
TtsT-No TT-in
TtsT-1 µg TT-in
TtsT-5 µg TT-in
TtsT-10 µg TT-in
TtsT-20 µg TT-in
TtsT-40 µg TT-in
TtsT-0.5 µg TT+CT-in
TtsT-1 µg TT + CT-in
TtsT-5 µg TT + CT-in
TtsC-0.5 µg TT + CT-in
TtsT-20 µg-sc

Figure 13

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(54) **Lipide in wässriger Phase.**

(57) Die vorliegende Erfindung betrifft ein neues, vorteilhaftes Verfahren zur Herstellung von unilamellaren Liposomen in wässriger Phase durch Ueberführen einer geeigneten Lipidkomponente, z.B. Phosphatidsäure, in die ionische Form, indem man die Lipiddispersion einer pH-Aenderung unterwirft und anschliessend neutralisiert. Die Bildung der unilamellaren Liposome erfolgt spontan d.h. ohne zusätzliche äussere Energiezufuhr. Die verfahrensgemäss erhältlichen Liposome können als Träger von Wirkstoffen unterschiedlichster Art therapeutisch verwendet werden.

EP 0 088 046 A2

CIBA-GEIGY AG
Basel (Schweiz)

4-13808/1+2

Lipide in wässriger Phase

Gegenstand der vorliegenden Erfindung ist ein Verfahren zur Herstellung von unilamellaren Liposomen in wässriger Phase.

Liposomen sind in der Literatur in zahlreichen Veröffentlichungen beschrieben worden. Ihr Aufbau und ihre Verwendung ist Gegenstand vieler Untersuchungen. Man unterscheidet unilamellare Liposomen mit einer Doppelschicht aus Lipiden von multilamellaren Liposomen mit mehreren Doppelschichten aus Lipiden, die zwiebelförmig angeordnet sind.

Unilamellare Liposomen haben eine kugelförmige Hülle und beispielsweise einen Durchmesser von ca. 200 bis 50000 Å, vorzugsweise ca. 200 bis 30000 Å. Die kugelförmige Hülle besteht aus einer Doppelschicht der Lipidkomponenten, z.B. amphipatischen Lipiden, z.B. Phospholipiden, z.B. Phosphatidsäure, Lecithin oder Kephalin, und gegebenenfalls neutralen Lipiden, z.B. Cholesterin. Diese Doppelschicht umschliesst einen Innenraum, der eine wässrige Phase enthält. Unilamellare Liposomen werden auch als "Vesikel" bezeichnet.

Es besteht grosses Interesse an der therapeutischen Verwendung von Liposomen als Träger von Wirkstoffen unterschiedlichster Art. So sind Liposomen als Träger von Proteinen, z.B. Antikörpern oder Enzymen, Hormonen, Vitaminen oder Genen oder zu analytischen Zwecken als Träger von markierten Verbindungen vorgeschlagen worden. Als Beispiel sei die US-Patentschrift 3,993,754 genannt, welche ein chemotherapeutisches Verfahren bei der Behandlung von Tumorzellen unter Verwendung von Liposomen als Träger zum Gegenstand hat.

Der betreffende Wirkstoff wird entweder bei der Bildung der Liposomen oder nachträglich durch Diffusion verkapselt. Die Herstellung von Liposomen und die Verkapselung des Wirkstoffs kann auf verschiedene Weise erfolgen und ist in dem Uebersichtsartikel "Liposomes-Problems and promise as selective drug carriers" von Kaye, St. B., Cancer Treatment Reviews (1981) 8, 27-50, beschrieben. Weitere Verfahren zur Herstellung von Liposomen zwecks Verkapselung von Wirkstoffen sind ebenfalls durch Barenholz et al. in Biochemistry, Vol 16, No. 12, 2806-2810, sowie in den Deutschen Offenlegungsschriften (DOS) 28 19 855, 29 02 672, 25 32 317 und 28 42 608, in der US-Patentschrift 4,053,585 und in der Europäischen Patentanmeldung 36 676 beschrieben.

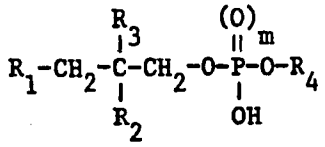
Man löst beispielsweise die Lipidkomponenten, z.B. Phospholipide, z.B. Phosphatidsäure, Lecithin oder Kephalin, und gegebenenfalls neutrale Lipide, z.B. Cholesterin, in einem organischen Lösungsmittel, z.B. Chloroform oder Benzol, auf. Nach dem Eindampfen bleibt eine homogene Schicht, z.B. eine Filmschicht, der betreffenden Lipidkomponenten zurück. Man dispergiert anschliessend die Lipidkomponenten in einer wässrigen Phase, welche den betreffenden Wirkstoff enthält, z.B. durch Schütteln. Bei der anschliessenden Behandlung mit Ultraschall bilden sich unilamellare Liposomen, welche den Wirkstoff verkapseln.

Nach vielen bisher bekannt gewordenen Verfahren erhält man wässrige Phasen sowohl mit Mischungen von unilamellaren als auch multilamellaren Liposomen, wobei Struktur und Grösse dieser Liposomen zufällig und kaum beeinflussbar sind und beträchtlich variieren können. Wässrige Phase mit überwiegendem Anteil an unilamellaren Liposomen erhält man bisher nur mit apparativ aufwendigen Herstellungsverfahren, z.B. durch Ultraschallbehandlung, Dialysieren oder Gelfiltration.

Nach dem Verfahren der vorliegenden Erfindung lassen sich auf einfache Weise wässrige Phasen mit einem hohen bis fast quantitativen Anteil an unilamellaren Liposomen herstellen, welche kleine unilamellare Liposomen (KUL) mit einem Durchmesser von ca. 200 - 600 Å und grosse unilamellare Liposomen (GUL) mit einem Durchmesser von ca. 600 - 3000 Å enthalten können. Ein besonderer Vorteil des erfindungsgemässen Verfahrens besteht darin, dass man KUL und GUL von relativ einheitlicher Grösse erhält und dass man das Mengenverhältnis von KUL zu GUL in der dispersen Phase variieren kann. Mittels geeigneter Trennmethoden, z.B. Gelfiltration oder einer Ultrafiltrationszelle, kann man kleine von grossen unilamellaren Liposomen abtrennen.

Die vorliegende Erfindung betrifft ein Verfahren zur Herstellung von unilamellaren Liposomen, dadurch gekennzeichnet, dass man

a) ein Lipid der Formel



(A),

worin m null oder ein ist, einer der Reste R_1 und R_2 Wasserstoff, Hydroxy, Niederalkyl mit 1-4 C-Atomen und der andere Rest Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff oder Niederalkyl mit 1-4 C-Atomen und R_4 Wasserstoff, Niederalkyl mit 1-7 C-Atomen, einen Kohlehydratrest mit 5-12 C-Atomen oder, wenn R_1 und R_2 Wasserstoff oder Hydroxy und R_3 Wasserstoff bedeuten, einen Steroidrest bedeuten, und ein geeignetes zusätzliches Lipid und/oder eine Fettsäure und ein geeignetes zusätzliches Lipid mit Ausnahme eines Sterins oder

ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 und R_4 Wasserstoff bedeuten und gegebenenfalls ein geeignetes zusätzliches Lipid

in wässriger Phase mit einem pH-Wert grösser als 7 dispergiert, oder

b) ein Lipid der Formel A, worin m null oder eins ist, einer der Reste R_1 und R_2 Wasserstoff, Hydroxy, Niederalkyl mit 1-4 C-Atomen und der andere Rest Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammoniogruppe substituiertes Niederalkyl bedeuten, und gegebenenfalls ein geeignetes zusätzliches Lipid oder

ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammonioniederalkylammoniogruppe substituiertes Niederalkyl bedeuten, und ein geeignetes zusätzliches Lipid in wässriger Phase mit einem pH-Wert kleiner als 7 dispergiert, und, wenn notwendig, die wässrige Phase neutralisiert und, wenn erwünscht, die erhältlichen unilamellaren Liposomen anreichert und/oder abtrennt.

Die weiter vorn und im folgenden verwendeten allgemeinen Begriffe haben im Rahmen der vorliegenden Beschreibung vorzugsweise die folgenden Bedeutungen:

Verfahren a)

Niederalkyl R_1, R_2 oder R_3 mit 1-4 C-Atomen ist z.B. bevorzugt Methyl, ferner Aethyl, n-Propyl, oder n-Butyl.

Alkyl R_1 oder R_2 ist vorzugsweise n-Decyl, n-Undecyl, n-Dodecyl (Lauryl), n-Tridecyl, n-Tetradecyl (Myristyl), n-Pentadecyl, n-Hexadecyl (Cetyl), n-Octadecyl (Stearyl) oder n-Eicosyl (Arachinyl), ferner n-Heptadecyl oder n-Nonadecyl.

n-Heptadecyl oder n-Nonadecyl.

Alkenyl R_1 oder R_2 ist vorzugsweise 9-cis-Dodecenyl (Lauroleyl), 9-cis-Tetradecenyl (Myristoleyl), 9-cis-Hexadecenyl (Palmitoleinyl), 6-cis-Octadecenyl (Petroselinyl), 6-trans-Octadecenyl (Petroselaidinyl), 9-cis-Octadecenyl (Oleyl), 9-trans-Octadecenyl (Elaidinyl) oder 9-cis-Eicosenyl (Gadoleinyl), ferner 1-Decenyl, 1-Undecenyl, 1-Dodecenyl, 1-Tridecenyl, 1-Tetradecenyl, 1-Pentadecenyl, 1-Hexadecenyl, 1-Heptadecenyl, 1-Octadecenyl, 9-cis-12-trans-Octadecadienyl (Linolyl), 9-trans-12-trans-Octadecadienyl (Linolaidinyl), 9-cis-12-cis-Octadienyl (Linoleyl), 9-cis-11-trans-13-trans-Octadecatrienyl (β-Eläostearinyl), 9-cis-12-cis-15-cis-Octadecatrienyl (Linolenyl), 9-, 11-, 13- 15-Octadecatetraenyl (Parinaryl), 1-Nonadecencyl, 1-Eicosenyl, 5-, 11-, 14-Eicosatrienyl oder 5-, 8-, 11-, 14-Eicosatetraenyl (Arachidonyl).

Alkoxy R_1 oder R_2 ist vorzugsweise n-Decyloxy, n-Dodecyloxy (Lauryloxy), n-Tetradecyloxy (Myristyloxy), n-Hexadecyloxy (Cetyloxy), n-Octadecyloxy (Stearyloxy) oder n-Eicosyloxy (Arachinyloxy), ferner n-Undecyloxy, n-Tridecyloxy, n-Pentadecyloxy, n-Heptadecyloxy oder n-Nonadecyloxy.

Alkenyloxy R_1 oder R_2 ist vorzugsweise 9-cis-Dodecenyloxy (Lauroleyloxy), 9-cis-Tetradecenyloxy (Myristoleyloxy), 9-cis-Hexadecenyloxy (Palmitoleinyloxy), 6-cis-Octadecenyloxy (Petroselinyloxy), 6-trans-Octadecenyloxy (Petroselaidinyloxy), 9-cis-Octadecenyloxy (Oleyloxy), 9-trans-Octadecenyloxy (Elaidinyloxy) oder 9-cis-Eicosenyl (Gadoleinyloxy), ferner 1-Decenyloxy, 1-Undecenyloxy, 1-Dodecenyloxy, 1-Tridecenyloxy, 1-Tetradecenyloxy, 1-Pentadecenyloxy, 1-Hexadecenyloxy, 1-Heptadecenyloxy, 1-Octadecenyloxy, 9-cis-12-trans-Octadecadienyloxy (Linolyloxy), 9-trans-12-trans-Octadecadienyloxy (Linolaidinyloxy), 9-cis-12-cis-Octadienyloxy (Linoleyloxy), 9-cis-11-trans-13-trans-Octadecatrienyloxy (β-Eläostearinyloxy), 9-cis-12-cis-15-cis-Octadecatrienyloxy (Linolenyloxy), 9-, 11-, 13-, 15-Octadecatetraenyloxy (Parinaryloxy), 1-Nonadecenyloxy, 1-Eicosenyloxy, 5-, 11-, 14-Eicosatrienyloxy oder 5-, 8-, 11-, 14-Eicosatetraenyloxy (Arachidonyloxy).

Acyloxy R_1 oder R_2 mit je 10-50 C-Atomen ist beispielsweise Alkanoyloxy, durch ein aromatisches Ringsystem substituiertes Alkanoyloxy oder Alkenoyloxy.

Alkanoyloxy R_1 oder R_2 ist vorzugsweise n-Decanoyloxy, n-Dodecanoyloxy (Lauroyloxy), n-Tetradecanoyloxy (Myristoyloxy), n-Hexadecanoyloxy, n-Hexadecanoyloxy (Palmitoyloxy), n-Octadecanoyloxy (Stearoyloxy) oder n-Eicosoyloxy (Arachinoyloxy), ferner n-Undecanoyloxy, n-Tridecanoyloxy, n-Pentadecanoyloxy, n-Heptadecanoyloxy oder n-Nonadecanoyloxy.

Durch ein aromatisches Ringsystem substituiertes Alkanoyloxy R_1 oder R_2 ist beispielsweise Phenyl-n-alkanoyloxy, worin der Phenylrest sich in ω-Stellung des Alkanoyloxyrests befindet, z.B. Phenyl-n-butyryloxy, -n-pentanoyloxy, -n-hexanoyloxy, -n-heptanoyloxy, -n-octanoyloxy, -n-nonanoyloxy, -n-decanoyloxy, -n-undecanoyloxy oder Phenyl-n-dodecanoyloxy, 3- oder 4-, vorzugsweise 4-Alkylphenyl-n-alkanoyloxy, worin der Alkylphenylrest sich in ω-Stellung des Alkanoyloxyrests befindet, z.B. 4-n-Butyl-, 4-n-Pentyl-, 4-n-Hexyl-, 4-n-Octyl-, 4-n-Decyl- oder 4-n-Dodecylphenyl-n-butyryloxy, -n-pentanoyloxy-, n-hexanoyloxy, -n-octanoyloxy, -n-decanoyloxy oder -n-dodecanoyloxy, Pyren-1-yl-n-alkanoyloxy, worin der Pyrenrest sich in ω-Stellung des Alkanoyloxyrests befindet, z.B. Pyren-1-yl-n-butyryloxy, -n-pentanoyloxy, -n-hexanoyloxy, -n-octanoyloxy, -n-decanoyloxy oder Pyren-1-yl-decanoyloxy, oder 6- oder 8-Alkylpyren-1-yl-n-alkanoyloxy, worin der Alkylpyren-1-ylrest sich in ω-Stellung des Alkanoyloxyrests befindet, z.B. 6- oder 8-Niederalkyl-, z.B. 6- oder 8-Aethylpyren-1-yl-n-butyryloxy, -n-pentanoyloxy, -n-hexanoyloxy, -n-octanoyloxy, -n-decanoyloxy oder -n-decanoyloxy, oder 6- oder 8-n-Butylpyren-1-yl-n-butyryloxy, -n-pentanoyloxy, -n-hexanoyloxy, -n-octanoyloxy, n-decanoyloxy oder -n-dodecanoyloxy, oder 6- oder 8-Alkylpyren-1-yl-n-alkanoyloxy, z.B. 6- oder 8-n-Decyl-, -n-Dodecyl-, -n-Tetradecyl-, -n-Hexadecyl- oder 6- oder 8-n-Octadecylpyren-1-yl-n-butyryloxy, -n-pentanoyloxy, n-hexanoyloxy, n-octanoyloxy, -n-decanoyloxy oder -n-dodecanoyloxy.

Durch ein aromatisches Ringsystem substituiertes Alkanoyloxy R_1 oder R_2 ist vorzugsweise 4-(4-n-Decylphenyl)-decanoyl, 4-(Pyren-1-yl)-butanoyl, 6-(Pyren-1-yl)-hexanoyl, 8-(Pyren-1-yl)-octanoyl, 10-(Pyren-1-yl)-octanoyl, 6-(6- oder 8-Aethylpyren-1-yl)-octanoyl, 6-(6- oder 8-n-Butylpyren-1-yl)-hexanoyl und 10-(6- oder 8-n-Octadecylpyren-1-yl)-decanoyl.

Alkenoyloxy R_1 oder R_2 ist vorzugsweise 9-cis-Dodecenyloxy (Lauroleoyloxy), 9-cis-Tetradecenoyloxy (Myristoleoyloxy), 9-cis-Hexadecenoyloxy (Palmitoleinoyloxy), 6-cis-Octadecenoyloxy (Petroselinoyloxy), 6-trans-Octadecenoyloxy (Petroselaidinoyloxy), 9-cis-Octadecenoyloxy (Oleoyloxy), 9-trans-Octadecenoyloxy (Elaidinoyloxy) oder 9-cis-Eicosenoyl (Gadoleinoyloxy), ferner 9-cis-12-trans-Octadienoyloxy (Linoloyl), 9-trans-12-trans-Octadecadienoyloxy (Linolaidinoyloxy), 9-cis-12-cis-Octadienoyloxy (Linoleoyloxy), 9-cis-11-trans-13-trans-Octadecatrienoyloxy (Linolenoyloxy), 9-, 11-, 13-, 15-Octadecatetraenoyloxy (Parinaroyloxy), 5-, 11-, 14-Eicosatrienoyloxy oder 5-, 8-, 11-, 14-Eicosatetraenoyloxy (Arachidonoyloxy).

Niederalkyl R_4 mit 1-7 C-Atomen ist z.B. Methyl, Aethyl, Isopropyl, n-Propyl, Isobutyl oder n-Butyl, und kann durch saure Gruppen, z.B. Carboxyl oder Sulfo, basische Gruppen, z.B. Amino, Niederalkylamino, z.B. Methyl- oder Aethylamino, Diniederalkylamino, z.B. Dimethyl- oder Diäthylamino, saure und basische Gruppen, z.B. Carboxy und Amino, wobei die Aminogruppe sich in α-Stellung zur Carboxylgruppe befindet, eine Triniederalkylammoniogruppe, z.B. Trimethyl- oder Triäthylammonio, freie oder verätherte Hydroxygruppen, wobei zwei verätherte Hydroxygruppen durch einen bivalenten Kohlenwasserstoffrest, z.B. durch Methylen, Aethylen, Aethyliden, 1,2-Propylen oder 2,2-Propylen, miteinander verbunden sein können, Halogen, z.B. Chlor oder Brom, Niederalkoxycarbonyl, z.B. Methoxy- oder Aethoxycarbonyl, oder durch Niederalkansulfonyl, z.B. Methansulfonyl, substituiert sein.

Substituiertes Niederalkyl R_4 mit 1-7 C-Atomen ist vorzugsweise Carboxyniederalkyl, z.B. Carboxymethyl, 2-Carboxyäthyl oder 3-Carboxy-n-propyl, Aminoniederalkyl, z.B. Aminomethyl, 2-Aminoäthyl oder 3-Amino-n-propyl, Niederalkylaminoniederalkyl, z.B. Methyl- oder Aethylaminomethyl, 2-Methylaminoäthyl oder 3-Methylamino-n-propyl, Diniederalkylaminoniederalkyl, z.B. Dimethyl- oder Diäthylaminomethyl, 2-Dimethylaminoäthyl oder 3-Dimethylamino-n-propyl, ω-Amino-ω-carboxyniederalkyl, z.B. 2-Amino-2-carboxyäthyl oder 3-Amino-3-carboxy-n-propyl, Triniederalkylammonioniederalkyl, z.B. 2-Trimethyl- oder 2-Triäthylammonioäthyl oder 3-Trimethyl- oder 3-Triäthylammonio-n-propyl, Hydroxyniederalkyl, z.B. 2-Hydroxyäthyl oder 2,3-Dihydroxypropyl, Niederalkoxyniederalkyl, z.B. Methoxy- oder Aethoxymethyl, 2-Methoxyäthyl oder 3-Methoxy-n-propyl, Niederalkylendioxyniederalykl, z.B. 2,3-Aethylendioxypropyl oder 2,3-(2,2-Propylen)-dioxypropyl, oder Halogenniederalkyl, z.B. Chlor oder Brommethyl, 2-Chlor- oder 2-Bromäthyl, 2- oder 3-Chlor- oder 2- oder 3-Brom-n-propyl.

Ein Kohlenhydratrest R_4 mit 5-12 C-Atomen ist beispielsweise ein natürlicher Monosaccharidrest, der sich von einer als Aldose oder Ketose vorliegenden Pentose oder Hexose ableitet.

Eine als Aldose vorliegende Pentose ist z.B. D-Ribose, D-Arabinose, D-Xylose oder D-Lyxose.
Eine als Ketose vorliegende Pentose ist z.B. D-Ribulose oder D-Xylulose.
Eine als Aldose vorliegende Hexose ist z.B. D-Allose, D-Altrose, D-Glucose, D-Mannose, D-Galactose oder D-Talose.
Eine als Ketose vorliegende Hexose ist z.B. D-Psicose, D-Fructose, D-Sorbose oder D-Tagatose.
Eine Hexose liegt vorzugsweise in zyklischer Form vor, z.B. als Pyranose (Aldose), z.B. α- oder β-D-Glucopyranose, oder als Furanose, z.B. α- oder β-D-Fructofuranose. Der Pyranosylrest ist vorzugsweise durch die in 1- oder 6-Stellung und der Furanosylrest durch in 1- oder 5-Stellung befindliche Hydroxygruppe mit der Phosphatidylgruppe verestert.

Ein Kohlehydratrest R_4 mit 5-12 C-Atomen ist ferner ein natürlicher Disaccharidrest, z.B. ein aus zwei Hexosen gebildeter Disaccharidrest, der beispielsweise durch Kondensation von zwei Aldosen, z.B D-Glucose oder D-Galactose oder einer Aldose, z.B. D-Glucose mit einer Ketose, z.B. Fructose, gebildet wird. Aus zwei Aldosen gebildete Disaccharide, z.B. Lactose oder Maltose, sind vorzugsweise über die in 6-Stellung des betreffenden Pyranosylrests befindliche Hydroxygruppe mit der Phosphatidylgruppe verestert. Aus einer Aldose und einer Ketose gebildete Disaccharide, z.B. Saccharose, sind vorzugsweise über die in 6-Stellung des Pyranosylrests oder über die in 1-Stellung des Furanosylrests befindliche Hydroxygruppe mit der Phosphatidylgruppe verestert.

Ein Kohlehydratrest R_4 mit 5-12 C-Atomen ist ferner ein derivatisierter Mono- oder Disaccharidrest, worin beispielsweise die Aldehydgruppe und/oder ein oder zwei endständige Hydroxygruppen zu Carboxylgruppen oxydiert sind, z.B. ein D-Glucon-, D-Glucar- oder D-Glucoronsäurerest, welche vorzugsweise als zyklische Lactonreste vorliegen. Ebenso können in einem derivatisierten Mono- oder Disaccharidrest Aldehyd- oder Ketogruppen zu Hydroxygruppen reduziert sein, z.B. Inosit, Sorbit oder D-Mannit, oder Hydroxygruppen durch Wasserstoff, z.B. Desoxyzucker, z.B. 2-Desoxy-D-ribose, L-Rhamnose oder L-Fucose, oder durch Aminogruppen, z.B. Aminozucker, z.B. D-Glucosamin oder D-Galactosamin, ersetzt sein.

Ein Kohlehydrat R_4 kann ebenfalls ein durch Umsetzung eines der genannten Mono- oder Disaccharide mit einem starken Oxydationsmittel, z.B. Perjodsäure, gebildetes Spaltprodukt sein.

Ein Steroidrest R_4 ist beispielsweise ein Sterinrest, der über die in 3-Stellung des Steroidgerüsts befindliche Hydroxygruppe mit der Phosphatidylgruppe verestert ist.

Ein Sterinrest ist beispielsweise Lanosterin, Sitosterin, Koprostanol, Cholestanol, Glykocholsäure, Ergosterin oder Stigmasterin, vorzugsweise Cholesterin.

Wenn R_4 einen Steroidrest darstellt, sind R_1 und R_2 vorzugsweise Hydroxy und R_3 ist Wasserstoff.

Eine Fettsäure ist beispielsweise eine gesättigte oder ungesättigte aliphatische Carbonsäure mit 4 bis 26, bevorzugt 10 bis 20, Kohlenstoffatomen.

Eine gesättigte aliphatische Carbonsäure ist beispielsweise eine geradkettige aliphatische Carbonsäure mit 10 bis 20 Kohlenstoffatomen, z.B. Caprinsäure (C-10), Undecansäure (C-11), Laurinsäure (C-12), Tridecansäure (C-13), Myristinsäure (C-14), Pentadecansäure (C-15), Palmitinsäure (C-16), Margarinsäure (C-17), Stearinsäure (C-18), Nonadecansäure (C-19) oder Arachinsäure (C-20).

Eine gesättigte aliphatische Carbonsäure ist beispielsweise eine verzweigtkettige aliphatische Carbonsäure mit 10 bis 20 Kohlenstoffatomen, z.B. Isomyristinsäure (C-14), Isopalmitinsäure (C-16), Isostearinsäure (C-18).

Eine ungesättigte aliphatische Carbonsäure mit 10-20 Kohlenstoffatomen hat beispielsweise eine gerade Anzahl von Kohlenstoffatomen und bis zu fünf Doppelbindungen und ist beispielsweise Myristoleinsäure (C-14), Palmitoleinsäure (C-16), Palmitaleidinsäure (C-16), Petroselinsäure (C-16), Oelsäure (C-18), Elaidinsäure (C-18), Vaccensäure (C-18), Linolsäure (C-18), Linolelaidinsäure (C-18), Linolensäure (C-18), cis-Eicos-5-ensäure (C-20), cis-11-Eicosensäure, 11,14-Eicosadiensäure, 11-, 14-, 17-Eicosatriensäure, Arachidonsäure oder 5-, 8-, 11-, 14-, 17-Eicosapentaensäure.

Die betreffende Fettsäure kann in undissoziierter Form oder in Form eines Salzes, z.B. als Alkalimetall-, z.B. Natrium- oder Kaliumsalz, vorliegen.

Ein geeignetes zusätzliches Lipid ist beispielsweise ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 Wasserstoff oder Niederalkyl mit je 1-7 C-Atomen, einen Kohlehydratrest mit 5-12 C-Atomen oder einen Steroidrest bedeuten.

R_1, R_2 und R_3 haben die weiter vorn genannten Bedeutungen. R_4 ist ausserdem durch Triniederalkylammonio, z.B. Trimethylammonio, substituiertes Niederalkyl, z.B. 2-Trimethylammonioäthyl (Cholinyl).

Ein geeignetes zusätzliches Lipid ist vorzugsweise ein Lipid der Formel A, worin R_1 und R_2 Acyloxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl oder 2-Aminoäthyl darstellen.

Ein solches zusätzliches Lipid ist z.B. ein natürliches Lecithin, z.B. Ei-Lecithin oder Lecithin aus Sojabohnen, wenn R_4 2-Trimethylammonioäthyl bedeutet, und ein natürliches Kephalin, z.B. Ei-Kephalin oder Kephalin aus Sojabohnen, wenn R_4 2-Aminoäthyl bedeutet.

Ausserdem sind als zusätzliche Lipide synthetische Lecithine (R_4 = 2-Trimethylammonioäthyl) und synthetische Kephaline (R_4 = 2-Aminoäthyl) der Formel A bevorzugt, worin R_1 und R_2 identische Acyloxyreste, z.B. Lauroyloxy, Oleoyloxy, Linoyloxy, Linoleoyloxy oder Arachinoyloxy bedeuten, z.B. Dilauroyl-, Dimyristoyl-, Dipalmitoyl-, Distearoyl-, Diarachinoyl-, Dioleoyl-, Dilinoyl-, Dilinoleoyl-, oder Diarachinoyllecithin oder -kephalin, R_1 und R_2 verschiedene Acyloxyreste, z.B. R_1 Palmitoyloxy und R_2 Oleoyloxy, z.B. 1-Palmitoyl-2-oleoyl-lecithin oder -kephalin, R_1 und R_2 identische Alkoxyreste, z.B. Tetradecyloxy oder Hexadecyloxy, z.B. Ditetradecyl- oder Dihexadecyllecithin oder -kephalin, R_1 Alkenyl und R_2 Acyloxy, z.B. ein Plasmalogen (R_4 = Trimethylammonioäthyl), oder R_1 Acyloxy, z.B. Myristoyloxy oder Palmitoyloxy, und R_2 Hydroxy, z.B. ein natürliches oder synthetisches Lysolecithin oder Lysokephalin, z.B. 1-Myristoyl- oder 1-Palmitoyllysolecithin oder -kephalin, und R_3 Wasserstoff darstellen.

Ein geeignetes zusätzliches Lipid ist ferner ein Lipid der Formel A, worin m eins ist, R_1 Alkenyl, R_2 Acylamido, R_3 Wasserstoff und R_4 einen 2-Trimethylammonioäthyl-Rest (Cholinrest) darstellen. Ein solches Lipid ist unter dem Namen Sphingomyelin bekannt.

Ein geeignetes zusätzliches Lipid ist ausserdem ein Lysolecithin-Analoges, z.B. 1-Lauroyl-1,3-propandiol-3-phosphorylcholin, ein Monoglycerid, z.B. Monoolein oder Monomyristin, ein Cerebrosid, ein Gangliosid oder ein Glycerid, welches keine freie oder verätherte Phosphoryl- oder Phosphonylgruppe in 3-Stellung enthält. Ein solches Glycerid ist beispielsweise ein Diacylglycerid oder 1-Alkenyl-1-hydroxy-2-acylglycerid mit den genannten Acyl- bzw. Alkenylgruppen, worin die 3-Hydroxygruppe durch einen der genannten Kohlehydratreste, z.B. einen Galactosylrest, veräthert ist, z.B. ein Monogalactosyl-glycerin.

Ein zusätzliches Lipid ist ferner ein neutrales Lipid, welches in Zellmembranen enthalten und nur in apolaren organischen Lösungsmitteln, z.B. in Chloroform, löslich ist. Neutrale Lipide sind beispielsweise Steroide, z.B. Oestradiol oder Sterine, z.B. Cholesterin, β-Sitosterin, Desmosterin, 7-Keto-Cholesterin oder β-Cholestanol, fettlösliche Vitamine, z.B. Vitamin A, z.B. Vitamin A_1 oder A_2, Vitamin E, Vitamin K, z.B. Vitamin K_1 oder K_2, Vitamin D_2 oder D_3, oder ein beliebiges Protein.

Bevorzugt enthält die wässrige Dispersion ein Lipid der Formel A, worin m eins ist, R_1 Alkyl, z.B. n-Dodecyl (Lauryl), n-Tridecyl, n-Tetradecyl (Myristyl), n-Pentacedyl, n-Hexadecyl (Cetyl), n-Heptadecyl oder n-Octadecyl (Stearyl), Alkoxy, z.B. n-Dodecyloxy (Lauryloxy), n-Tetradecyloxy (Myristyloxy), n-Hexadecyloxy (Cetyloxy), oder n-Octadecyloxy (Stearyloxy), Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_2 Wasserstoff oder Hydroxy, R_3 Wasserstoff oder Niederalkyl, z.B. Methyl, und R_4 Wasserstoff, Niederalkyl, z.B. Methyl oder Aethyl, Niederalkyl substituiert durch saure und

basische Gruppen, z.B. Carboxy und Amino, z.B. ω-Amino-ω-carboxyniederalkyl, z.B. 2-Amino-2-carboxyäthyl oder 3-Amino-3-carboxy-n-propyl, Hydroxyniederalkyl, z.B. 2-Hydroxyäthyl oder 2,3-Hydroxypropyl, Niederalkylendioxyniederalkyl, z.B. 2,3-Aethylendioxypropyl oder 2,3-(2,2-Propylen)-dioxypropyl, Halogenniederalkyl, z.B. 2-Chlor- oder 2-Bromäthyl, einen Kohlehydratrest mit 5-12 C-Atomen, z.B. Inosit, oder einen Steroidrest, z.B. ein Sterin, z.B. Cholesterin bedeuten, und ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl oder 2-Aminoäthyl bedeuten. Die wässrige Dispersion kann auch bevorzugt ein Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 Wasserstoff bedeuten, und gegebenenfalls ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl, 2-Aminoäthyl, Niederalkyl substituiert durch saure und basische Gruppen, z.B. Carboxy und Amino, z.B. ω-Amino-ω-carboxyniederalkyl, z.B. 2-Amino-2-carboxyäthyl oder 3-Amino-3-carboxy-n-propyl, oder einen Kohlehydratrest mit 5-12 C-Atomen bedeuten, z.B. Inosit oder ein Monoglycerid, z.B. Monoolein oder Monomyristin, oder ein Sterin, z.B. Cholesterin, enthalten.

In erster Linie enthält die wässrige Dispersion eine Lysophosphatidsäure, z.B. eine natürliche Lysophosphatidsäure, z.B. Ei-Lysophosphatidsäure, oder eine synthetische Lysophosphatidsäure, z.B. 1-Lauroyl-, 1-Myristoyl- oder 1-Palmitoyllysophosphatidsäure, ein Lysophosphatidylserin, z.B. ein natürliches Lysophosphatidylserin, z.B. Lysophosphatidylserin aus dem Rinderhirn, oder ein synthetisches Lysophosphatidylserin, z.B. 1-Myristoyl- oder 1-Palmitoyllysophosphatidylserin, ein Lysophosphatidylglycerin oder ein Lysophosphatidylinositol und zusätzlich ein Lecithin, z.B. ein natürliches Lecithin, z.B. Ei-Lecithin, oder ein Lecithin mit gleichen Acyloxygruppen, z.B. Dimyristoyl- oder Dipalmitoyllecithin, ein Lecithin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoyllecithin, oder zusätzlich ein Kephalin, z.B. ein

natürliches Kephalin, z.B. Ei-Kephalin, oder ein Kephalin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoylkephalin.

In erster Linie kann die wässrige Dispersion auch eine natürliche Phosphatidsäure, z.B. Ei-Phosphatidsäure, eine synthetische Phosphatidsäure, z.B. Dilauroyl-, Dimyristoyl-, Dipalmitoyl- oder 1-Palmitoyl-2-oleoylphosphatidsäure, und gegebenenfalls zusätzlich ein Lecithin, z.B. ein natürliches Lecithin, z.B. Ei-Lecithin, ein Lecithin mit gleichen Acyloxygruppen, z.B. Dimyristoyl- oder Dipalmitoyllecithin, oder ein Lecithin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoyllecithin, oder ein Kephalin, z.B. ein natürliches Kephalin, z.B. Ei-Kephalin oder ein Kephalin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoylkephalin, oder ein Phosphatidylserin, z.B. ein natürliches Phosphatidylserin, z.B. Phosphatidylserin aus dem Rinderhirn, oder ein synthetisches Phosphatidylserin, z.B. Dipalmitoylphosphatidylserin, ein Monoglycerid, z.B. Monoolein oder Monomyristin, oder ein Sterin, z.B. Cholesterin, enthalten.

Zur Herstellung von unilamellaren Liposomen stellt man zunächst eine homogene Schicht der Lipidkomponenten her. Die Herstellung der homogenen Schicht kann in an sich bekannter Weise erfolgen und ist weiter hinten im Abschnitt "Herstellung der homogenen Schicht der Lipidkomponenten" beschrieben.

Die homogene Schicht dispergiert man in wässriger Phase und erhöht anschliessend den pH-Wert von solchen wässrigen Phasen, worin nur eine Lipidkomponente, z.B. reine Phosphatidsäure, dispergiert ist, bis auf ca. 12, bevorzugt bis auf ca. 9 bis 11. Dies erfolgt beispielsweise durch Zugabe von physiologisch annehmbaren, basischen Lösungen, z.B. verdünnter wässriger, ca. 0,01 - 0,2 N, insbesondere ca. 0,1 N Natriumhydroxid- oder Kaliumhydroxid-Lösung, unter gleichzeitiger Kontrolle des pH-Werts. z.B. durch Tüpfelprobe oder ein pH-Meter. In wässrigen Phasen, worin mehrere Lipidkomponenten, z.B. Phophatidsäure und Lecithin, dispergiert sind, reicht eine Erhöhung des pH-Werts auf

ca. 8-9 aus. Man kann diesen pH-Bereich ebenfalls durch Zugabe von wässrigen Basen, z.B. verdünnter Natronlauge oder Kalilauge, unter gleichzeitiger pH-Kontrolle oder durch Zugabe von Pufferlösung, z.B. Phosphatpufferlösung mit einem geeigneten pH-Wert von 7 bis 8, einstellen.

In einer bevorzugten Ausführungsform dispergiert man die homogene Schicht der Lipidkomponenten in wässrigen Phasen mit einem pH-Wert grösser als 7, z.B. in physiologisch annehmbaren, basischen Lösungen, z.B. in verdünnter wässriger, ca. 0,01 - 0,2 N, insbesondere 0,1 N Natriumhydroxid- oder Kaliumhydroxid-Lösung. Eine Lipidkomponente, z.B. reine Phosphatidsäure, dispergiert man in wässrigen Phasen mit einem pH-Wert bis ca. 12, bevorzugt ca. 9 bis 11. Mehrere Lipidkomponenten, z.B. Phosphatidsäure und Lecithin, dispergiert man in wässrigen Phasen mit einem pH-Wert von ca. 8 bis 9.

Verfahren b)

Für ein Lipid der Formel A, worin m null oder eins ist, einer der Reste R_1 und R_2 Wasserstoff, Hydroxy, Niederalkyl mit 1-4 C-Atomen und der andere Rest Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammoniogruppe substituiertes Niederalkyl bedeuten, haben R_1 und R_2 die weiter vorn unter Verfahren a) genannten Bedeutungen.

Durch eine Ammoniogruppe substituiertes Niederalkyl R_4 ist beispielsweise durch eine Triniederalkylammoniogruppe, z.B. Trimethyl- oder Triäthylammonio, substituiertes Niederalkyl, z.B. 2-Trimethyl- oder 2-Triäthylammonioäthyl.

Für ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammonioniederalkylammoniogruppe substituiertes Niederalkyl bedeuten, haben R_1 und R_2 die weiter vorn unter Verfahren a) genannten Bedeutungen.

Durch eine Ammonioniederalkylammoniogruppe substituiertes Niederalkyl ist beispielsweise 2-[N,N-Diniederalkyl-N-(2-N',N',N'-triniederalkylammonioäthyl)-ammonio]-äthyl, z.B. 2-[N,N-Dimethyl-N-(2-N',N',N'-trimethylammonioäthyl)-ammonio]-äthyl.

Ein geeignetes zusätzliches Lipid ist eins der weiter vorn unter Verfahren a) genannten zusätzlichen Lipide.

Bevorzugt enthält die wässrige Dispersion ein Lipid der Formel A, worin m eins ist, R_1 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_2 Hydroxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl bedeuten, und ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Aminoäthyl oder 2-Trimethylammonioäthyl bedeuten. Die wässrige Dispersion kann auch bevorzugt ein Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_2 2-[N,N-Dimethyl-N-(2-N',N',N'-trimethylammonioäthyl)-ammonio]-äthyl bedeuten und gegebenenfalls ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Aminoäthyl oder 2-Trimethylammonioäthyl bedeuten, enthalten.

In erster Linie enthält die wässrige Dispersion ein Lysophosphatidylcholin (Lysolecithin) und ein natürliches Lecithin, z.B. Ei-Lecithin. In erster Linie kann die wässrige Dispersion auch ein Phosphatidyl-2-[N,N-Dimethyl-N-(2-N',N',N'-trimethylammonioäthyl)-ammonio]-äthylchlorid und gegebenenfalls ein natürliches Lecithin, z.B. Ei-Lecithin, enthalten.

Zur Herstellung von unilamellaren Liposomen stellt man zunächst eine homogene Schicht der Lipidkomponenten, z.B. Lysolecithin oder Phophatidyl-2-[N,N-Dimethyl-N-(2-N',N',N'-trimethylammonioäthyl)-ammonio]-äthylchlorid, her.

Die Herstellung der homogenen Schicht kann in an sich bekannter Weise erfolgen und ist weiter hinten im Abschnitt "Herstellung der homogenen Schicht der Lipidkomponenten" beschrieben.

Die homogene Schicht dispergiert man in wässriger Phase und erniedrigt anschliessend den pH-Wert bis auf ca. 1 oder darunter unter gleichzeitiger Kontrolle des pH-Werts, z.B. durch Tüpfelproben oder ein pH-Meter. Dies erfolgt beispielsweise durch Zugabe von physiologisch annehmbaren Säuren, beispielsweise verdünnten wässrigen Mineralsäuren, z.B. verdünnter wässriger Schwefelsäure, Salzsäure oder Phosphorsäure.

In einer bevorzugten Ausführungsform dispergiert man die homogene Schicht der Lipidkomponenten in wässrigen Phasen mit einem pH-Wert von ca. 1 oder Werten darunter, z.B. in verdünnnten wässrigen Mineralsäuren, z.B. verdünnter wässriger Schwefelsäure, Salzsäure oder Phosphorsäure unter gleichzeitiger Kontrolle des pH-Werts.

Eine anschliessende Neutralisierung der wässrigen Phasen ist notwendig, wenn man zuvor den pH-Wert der wässrigen Phase gemäss Verfahren a) auf Werte höher als 8 oder gemäss Verfahren b) niedriger als 5 eingestellt hat. Dies erfolgt, um unmittelbar nach der pH-Wert Erniedrigung oder Erhöhung eine Zerstörung des Wirkstoffs und/oder der Liposomen unter basischen bzw. sauren Bedingungen zu vermeiden. Die basisch gemachte wässrige Phase neutralisiert man mit einer geeigneten physiologisch annehmbaren Säure oder einer Pufferlösung, z.B. Phosphatpufferlösung mit einem pH-Wert von 7 bis 8. Geeignete Säuren sind beispielsweise die weiter vorn genannten verdünnten wässrigen Mineralsäuren sowie schwache organische Säuren, z.B. Ameisensäure oder Essigsäure. Die saure wässrige Phase neutralisiert man durch Zugabe von wässrigen Basen, z.B. verdünnter wässriger Natrium- oder Kaliumhydroxid-Lösung. Man neutralisiert unter gleichzeitiger Kontrolle des pH-Werts.

Die Lipide sind in Konzentrationen bis über 70% in der wässrigen Phase dispergiert. Der Konzentrationsbereich von ca. 1% bis ca. 20% ist bevorzugt.

Man arbeitet zweckmässigerweise bei Raumtemperatur oder höheren Temperaturen, z.B. bis ca. 60°C. Falls es die Empfindlichkeit des zu verkapselnden Wirkstoffs verlangt, führt man das Verfahren unter Kühlen und gegebenenfalls in einer Inertgasatmosphäre, z.B. Stickstoffatmosphäre, durch.

Sowohl nach Verfahren a) als auch nach Verfahren b) findet die Bildung von unilamellaren Liposomen spontan (spontaneous vesiculation), d.h. ohne zusätzliche Energiezufuhr von aussen und mit grosser Geschwindigkeit, statt.

Die nach Verfahren a) und b) erhältlichen unilamellaren Liposomen sind in wässriger Phase relativ lange stabil. Beispielsweise bleiben unilamellare Liposomen bestehend aus Ei-Phosphatidsäure oder Ei-Phosphatidsäure und Ei-Lecithin in wässeriger Phase bei 4°C gelagert mehr als 14 Tage lang stabil. Wässrige Phasen mit erfindungsgeäss herstellbaren unilamellaren Liposomen können nach den in der Europäischen Patentanmeldung O 065 292 angegebenen Verfahren lagerungsfähig gemacht werden.

Die erfolgte Bildung von unilamellaren Liposomen und ihr Gehalt in wässriger Phase lassen sich in an sich bekannter Weise anhand verschiedener Messmethoden, z.B. optisch im Elektronenmikroskop, durch Massenbestimmung in der analytischen Ultrazentrifuge und vor allem spektroskopisch, z.B. im Kernresonanzspektrum (^{1}H, ^{13}C und ^{31}P), nachweisen. So geben beispielsweise scharfe Signale im Kernresonanzspektrum einen Hinweis auf erfolgte Bildung von kleinen unilamellaren Liposomen. Der Anteil an gebildeten kleinen unilamellaren Liposomen im System kann aus der Intensität der Signale berechnet werden. So ist im Protonen-Kernresonanzspektrum ein scharfes Methylensignal bei δ = 1,28 ppm und ein scharfes Methylsignal bei δ = 0,89 ppm für kleine unilamellare Liposomen, welche aus Phosphatidsäure gebildet werden, charakteristisch. Kleine unilamellare Liposomen, welche aus Phosphatidsäure und Lecithin bestehen, zeigen ebenfalls das Methylen- und das

Methylsignal bei δ= 1,28 und 0,89 ppm und zusätzlich ein Methylsignal bei δ= 3,23 ppm, welches der Trimethylammoniogruppe des Lecithins zugeordnet wird.

Die Grösse der gebildeten unilamellaren Liposomen ist u.a. von der Struktur der Lipidkomponenten, dem Mischungsverhältnis der Lipidkomponenten, der Konzentration dieser Lipidkomponenten in der wässrigen Phase und von der Menge und Struktur des zu verkapselnden Wirkstoffs abhängig. So kann man beispielsweise durch Variation der Konzentration der Lipidkomponenten wässrige Phasen mit einem hohen Anteil an kleinen oder grossen unilamellaren Liposomen herstellen. Beispielweise wird durch Zugabe von Ei-Phosphatidsäure zur dispersen Phase der Anteil an kleinen unilamellaren Liposomen (KUL) erhöht. Der Anteil von GUL in einer dispersen Phase lässt sich auch durch Zusatz von Salzen, z.B. NaCl oder KCl erhöhen. Der Durchmesser der beispielsweise aus Phosphatidsäure oder Phosphatidsäure und Lecithin gebildeten KUL beträgt ca. 200 - 600 Å. Das Einschlussvolumen für KUL von dieser Grösse beträgt ca. 0,5 bis 1 l pro Mol eingesetzter Lipidkomponente.

Zusätzlich zu KUL enstehen auch grosse unilamellare Liposomen (GUL-Durchmesser bis zu 50,000 Å). Diese schliessen grössere Volumina pro Mol eingesetzter Lipidkomponenten ein und eignen sich zur Verkapselung mit höherer Ausbeute und zum Einschluss von voluminösen Materialien, z.B. Viren, Bakterien oder Zellorganellen.

Die Trennung der KUL von GUL erfolgt mittels herkömmlicher Trennmethoden, z.B. Gelfiltration, z.B. mit Sepharose 4B als Träger, oder durch Sedimentation der GUL in der Ultrazentrifuge bei 160,000 x g. Beispielsweise setzen sich nach mehrstündigem, ca. dreistündigem, Zentrifugieren in diesem Schwerefeld die GUL ab, während die KUL dispergiert bleiben und dekantiert werden können. Nach mehrmaligem Zentrifugieren erreicht man eine vollständige Trennung der GUL von KUL.

Auch durch Gelfiltration kann man alle in der wässrigen Phase befindlichen Liposomen mit einem Durchmesser grösser als 600 Å, z.B. GUL oder multilamellare Liposomen, sowie nicht verkapselte Wirkstoffe und überschüssige, dispergierte Lipide abtrennen und so eine wässrige Phase mit einer Fraktion KUL von relativ einheitlicher Grösse erhalten.

Die erfindungsgemäss erhältlichen Liposomen (KUL und GUL) sind geeignete Trägersysteme, welche in wässriger Phase zur Solubilisierung von lipophilen Stoffen, z.B. fettlöslichen Farbstoffen, zur Stabilisierung von hydrolyseempfindlichen Stoffen, z.B. Prostaglandinen, zum Einschluss von Schädlingsbekämpfungsmitteln, z.B. zur Veränderung des Wirkungsprofils von Dichlorphos, zum Einschluss von Nahrungsmittelzusätzen, z.B. zwecks Aenderung des Adsorptionsverhaltens von Vitaminen oder Farbstoffen, oder zur Einschleusung von verkapselten Wirkstoffen, Enzymen, Antikörpern, Hormonen, Genen, Viren, Vitaminen oder Zellorganellen in die Zellen einer Zellkultur verwendet werden können.

Wässrige Phasen, welche die erfindungsgemäss erhältlichen Liposome mit verkapselten Wirkstoffen enthalten, sind Verabreichungssysteme, welche sich, gegebenenfalls nach Konzentrierung oder Isolierung der Liposomen, z.B. durch Ultrazentrifugieren, zu therapeutischen Zwecken für die orale (p.o.), parenterale (i.v., i.m. oder i.p.) oder topikale Verabreichung eignen.

Bei oraler Verabreichung können Verabreichungssysteme auf Liposomenbasis einen Wirkstoff, beispielsweise Insulin, das im Verdauungstrakt unbeständig ist, schützen oder seine Resorption verbessern. Für die orale Verabreichung kann die Liposomen-haltige wässrige Phase mit pharmazeutisch unbedenklichen Verdünnungsmitteln oder Trägern oder mit üblichen Zusätzen, z.B. Farbstoffen oder Geschmacksstoffen, vermischt und als Sirup oder in Form von Kapseln verabreicht werden.

Bei parenteraler Verabreichung können Verabreichungssysteme auf Liposomenbasis beispielsweise die Verweilzeit z.B. von Desferrioxamin, siehe Guilmette R.A. et al., Life Sci. 22 (4) 313-320, 1978, oder Gentamycin, siehe Scheld W.M. et al., Clin. Res. 26, No. 1, 59 A, 1978, in einem Organismus verlängern. Ebenso wird die Verweilzeit von verkapselten Chelatbildern, z.B. EDTA (Aethylendiamintetraessigsäure), in Organismen verlängert, so dass man durch Chelatbildung Schwermetalle besonders aus Leber, Milz oder Nieren entfernen kann, siehe Rahmann et al., Science, Vol. 180, 300-302, 1973, und J. Lab. Clin. Med. 640-647, 1974. Mit Verabreichungssystemen auf Liposomenbasis kann man Wirkstoffe im Myokard anreichern, siehe Landesmann et al., Science Vol. 198, 737-738, 1977. Antiinflammatorisch wirkende Stoffe, z.B. Cortisol, siehe Nature 271, No. 5643, 372-73, 1978, oder Proteaseinhibitoren, siehe Anal. Biochem. 89, No. 2, 400-07, 1978, kann man in der Gelenkflüssigkeit und Cytostatika in Tumorgewebe, siehe Uebersichtsartikel von Kaye St. B., Cancer Treatment Reviews 8, 27-50, 1981, und die vielen darin zitierten Literaturstellen, anreichern. Manche Chemotherapeutika in der Krebstherapie sind weniger toxisch und besser verträglich, wenn sie in Liposomen verkapselt verabreicht werden, z.B. liposomverkapseltes Actinomycin D, siehe Rahman et al., Proceedings of the Society for Experimental Biogoly and Medicine 146, 1173-1176, 1974, Methotrexat, siehe Lesermann L.D. et al. Proc. Natl. Acad. Sci. 77, No. 7, 4089-93, 1980, Vinblastin, Daunomycin oder Cytosin-Arabinosid, siehe Mühlensiepen et al., Cancer Res. 41, Nr. 5, 1602-07, 1981. Liposomen können zur Einschleusung von Wirkstoffen, z.B. Enzymen, Peptidhormonen, Genen oder Viren in das Cytoplasma von Zellen in lebenden Organismen, z.B. zur Einschleusung von Asparaginase, siehe Uebersichtsartikel von Finkelstein M. und Weissmann G., J. Lipid Research, Vol. 19, 1978, 289-303, von Amyloglucosidase, siehe Gregoriadis G. und Ryman B.E., Eur. J. Biochem. 24 (1972), 485-491, oder Neuraminidase, siehe Gregoriadis et al., Biochem. J. (1974) 140, 232-330, zur Verankerung spezifischer Erkennungsmoleküle, z.B. monoklonaler Antikörper, zwecks zielgerichteter Einschleusung in definierte Zielzellen, siehe Leserman et al., Nature 293 (5829), 226-228, 1981, zur

Immunstimulation als Adjuvans bei Impfungen, z.B. gegen Leishmaniasen, siehe New R.R.C. et al. Nature 272 (5648) 55-56, 1978, oder zur induzierten Freisetzung von Wirkstoffen durch Signale wie Temperaturerhöhungen, z.B. in entzündetem Gewebe, oder pH-Wert Aenderungen verwendet werden. Für die parenterale Verabreichung können die konzentrierten oder isolierten Liposomen in einer geeigneten Trägerflüssigkeit, beispielsweise in sterilem destilliertem Wasser oder in physiologischer Kochsalzlösung, suspendiert werden.

Herstellung der homogenen Schicht der Lipidkomponenten

Die Herstellung der homogenen Schicht der Lipidkomponenten kann in an sich bekannter Weise erfolgen. Beispielsweise löst man zunächst das Lipid oder Lipidgemisch der Formel A, z.B. reine Ei-Phosphatidsäure oder eine Mischung aus Ei-Phosphatidsäure und Ei-Lecithin, gegebenenfalls unter Zumischung eines lipophilen Wirkstoffs, z.B. Proteins, das bei der Bildung der Liposomen in der Lipidschicht, eingeschlossen wird, in einem organischen Lösungsmittel auf. Durch Entfernen des organischen Lösungsmittels, am zweckmässigsten im Vakuum oder durch Abblasen im Inertgas, z.B. Stickstoff, stellt man eine homogene Schicht der Lipidkomponenten her.

Die Auswahl des betreffenden Lösungsmittels ist von der Löslichkeit der betreffenden Lipidkomponenten darin abhängig. Geeignete Lösungsmittel sind beispielsweise unsubstituierte oder substituierte, z.B. halogenierte, aliphatische, cycloaliphatische, aromatische oder aromatisch-aliphatische Kohlenwasserstoffe, z.B. Benzol, Toluol, Methylenchlorid oder Chloroform, Alkohole, z.B. Methanol oder Aethanol, Niederalkancarbonsäureester, z.B. Essigsäureäthylester, Aether, z.B. Diäthyläther, Dioxan oder Tetrahydrofuran, oder Mischungen dieser Lösungsmittel.

Die in der Beschreibung der vorliegenden Erfindung erwähnten Lipide sind bekannt oder können, falls sie neu sind, in an sich bekannter Weise nach den im Standardwerk von Knight C.G. Liposomes, Elsevier 1981, Kapi-

tel 3, befindlichen Vorschriften hergestellt werden. Alle genannten Lipide können in der wässrigen Dispersion als optisch aktive Derivate oder als Racemate enthalten sein. Die folgenden Beispiele veranschaulichen die Erfindung, ohne sie zu beschränken. Temperaturen sind in ° Grad Celsius angegeben und Mischungsverhältnisse auf Volumina bezogen.

Beispiel 1:

a) Man löst 1 g Ei-Phosphatidsäure in 20 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum im Rotationsverdampfer ein. Man dispergiert den filmartigen Rückstand in 20 ml destilliertem Wasser durch fünf Minuten langes Schütteln, wobei sich ein pH-Wert von ca. 3 einstellt. Zur Bildung von unilamellaren Liposomen gibt man anschliessend zur dispersen Phase bei Raumtemperatur unter Kontrolle mit einem pH-Meter soviel 0,1 N Natriumhydroxid-Lösung, bis der pH-Wert auf 11 steigt. Der pH-Wert der wässrigen Phase wird anschliessend durch Zugabe von 0,1 N HCl von 11 bis auf ca. 7 gesenkt. Man erhält eine leicht opaleszierende, wässrige Phase.

Die gebildeten unilamellaren Liposomen können im Elektronenmikroskop sichtbar gemacht werden. Die Liposomendispersion wird zunächst der üblichen Gefrierbruchmethode (freeze-fracture) unterzogen. Es liegen hauptsächlich zwei "Populationen" von unilamellaren Liposomen vor, die sich durch ihre durchschnittliche Grösse unterscheiden:
1. Kleine unilamellare Liposomen (KUL) mit einem Durchmesser von ca. 200-600 Å und
2. Grosse unilamellare Liposomen (GUL) mit einem Durchmesser von ca. 1,000 - 10,000 Å.
KUL sind im Protonen-NMR-Spektrum durch die Signale δ = 1,28 (Methylen) und δ = 0,89 ppm (Methyl) erkennbar. Die Ausbeute an KUL kann aus den Intensitäten der Signale abgeschätzt werden und beträgt ca. 56 %.

b) Analog Beispiel 1a) löst man 4 mal je 10 mg Ei-Phosphatidsäure in 4 mal je 0,2 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den filmartigen Rückstand in 1 ml destilliertem Wasser durch 5 Minuten langes

Schütteln. Zur Bildung von unilamellaren Liposomen gibt man anschliessend zu jeder einzelnen dispersen Phase unter Kontrolle mit einem pH-Meter soviel 0,1 N Natriumhydroxid-Lösung bis ein pH-Endwert von jeweils 6, 8, 11,3 und 11,6 resultiert. Die Ausbeute an KUL beträgt mit ansteigendem pH-Wert für jede Probe 5, 24, 57 und 60 %.

Beispiel 2:

a) Man löst 1 g Ei-Phosphatidsäure in 20 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 50 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt ca. 100 %.

b) Analog Beispiel 2a) löst man 4 mal je 10 mg Ei-Phosphatidsäure in 4 mal je 0,2 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jede Probe in soviel 0,01 N Natriumhydroxid-Lösung und destilliertem Wasser unter Schütteln, dass sich pH-Werte von ca. 7,3, 8,0, 9,4 und 10,0 einstellen. Die Ausbeute an KUL beträgt mit ansteigendem pH-Wert für jede Probe 33, 46, 65 und 81 %.

Beispiel 3:

Man löst 0,1 g Dilauroylphosphatidsäure in 5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 50 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL (ca. 300 - 800 Å) beträgt 73 %.

Beispiel 4:

a) Man löst 3 mg Ei-Phosphatidsäure und 7 mg Ei-Lecithin in 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser durch fünf Minuten langes Schütteln bei Raumtemperatur, worauf sich ein pH-Wert von ca. 3 einstellt. Zur Bildung von unilamellaren Liposomen gibt man anschliessend bei Raumtemperatur unter Kontrolle mit einem pH-Meter soviel 0,1 Natronlauge, bis der pH-Wert auf ca. 11,2 steigt. Durch Zugabe von Phosphatpufferlösung stellt man anschliessend den pH-Wert der wässrigen Phase auf ca. 7 ein. Man erhält eine leicht opaleszierende, wässrige Phase. Die erfolgte Bildung von unilamellaren Liposomen ist im NMR-Spektrum durch die Signale δ= 1,28 (Methylen), δ= 0,89 (Methyl) und δ= 3,23 (N-CH_3) erkennbar. In der elektronenmikroskopischen Abbildung sind hauptsächlich zwei "Populationen" von unilamellaren Liposomen zu erkennen, die sich durch ihre durchschnittliche Grösse unterscheiden:

1. KUL mit einem Durchmesser von ca. 200 - 800 Å und
2. GUL mit einem Durchmesser von ca. 1000 - 10,000 Å.

Die Ausbeute an KUL beträgt 45 %.

b) Analog Beispiel 4a) löst man 2 mal je 3 mg Ei-Phosphatidsäure und 7 mg Ei-Lecithin in 2 mal je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den filmartigen Rückstand in 1,0 ml destilliertem Wasser durch 5 Minuten langes Schütteln. Zur Bildung von unilamellaren Liposomen gibt man anschliessend zu jeder einzelnen Phase unter Kontrolle mit einem pH-Meter soviel 0,1 N Natriumhydroxid-Lösung unter Schütteln, bis ein Endwert von 8,6 und 10 eingestellt ist. Die Ausbeute an KUL beträgt mit ansteigendem pH-Wert 22 und 35 %.

c) Analog Beispiel 4a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure und Ei-Lecithin in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den filmartigen Rückstand in 1,0 ml destilliertem Wasser durch 5 Minuten langes Schütteln. Zur Bildung von unilamellaren Liposomen gibt man anschliessend zu jeder einzelnen Phase unter Kontrolle mit einem pH-Meter soviel 0,1 N Natriumhydroxid-Lösung unter Schütteln, bis sich ein pH-Wert von ca. 11,2 eingestellt hat.
Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	6	10	14	20	25	30	33	50	48	60
%-KUL	5	9	14	17	19	20	27	39	41	50

Beispiel 5:

a) Man löst 0,3 g Ei-Phosphatidsäure und 0,7 g Ei-Lecithin in 10 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert diesen Rückstand in 10 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt ca. 30 %.

b) Analog Beispiel 5a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidlösung und Ei-Lecithin (insgesamt 10 mg Lipid) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert jeweils den filmartigen Rückstand in je 1 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	10	20	25	30	40	50	60	80
%-KUL	14	22	31	42	45	50	78	95

Beispiel 6:

a) Man löst 0,7 g Ei-Lecithin, 0,3 g Phosphatidylserin aus dem Rinderhirn und 2 g Ei-Phosphatidsäure in 20 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum im Rotationsverdampfer ein. Man dispergiert den filmartigen Rückstand in 100 ml 0,01 N Natriumhydroxid-Lösung durch fünf Minuten langes Schütteln bei Raumtemperatur, wobei sich ein pH-Wert von ca. 12 einstellt. Durch Zugabe von 1 N Salzsäure wird der pH-Wert der wässrigen Phase auf ca. 7 eingestellt. Man erhält eine leicht opaleszierende, wässrige Phase.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind GUL und KUL zu erkennen.

b) Analog Beispiel 6a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure, aber gleicher Menge an Ei-Lecithin und Phosphatidylserin (insgesamt 10 mg Lipid) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den Rückstand in je 1,0 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	9	10	26	33	34	40	60
%-KUL	14	18	26	36	47	43	64

Beispiel 7:

a) Man löst 1 g Asolectin (Phospholipidgemisch hauptsächlich bestehend aus Lecithin, Kephalin, Phosphatidylserin und Phosphatidylinosit) und 0,2 g Ei-Phosphatidsäure in 20 ml einer Chloroform/Methanol

Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 100 ml 0,01 N Natriumhydroxid-Lösung durch fünf Minuten langes Schütteln bei Raumtemperatur, wobei sich ein pH-Wert von ca. 12 einstellt. Durch Zugabe von 1 N Salzsäure wird der pH-Wert der wässrigen Phase auf ca. 7 gebracht. Man erhält eine leicht opaleszierende, wässrige Phase.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind GUL und KUL zu erkennen.

b) Analog zu Beispiel 6a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure, aber gleicher Menge an Asolectin (insgesamt 10 mg Lipid) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den Rückstand in je 1 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	17	37	50
%-KUL	24	69	65

Beispiel 8:

a) Man löst 0,1 g einer Mischung aus Ei-Lecithin und Cholesterin (Molverhältnis 1:1) und 0,1 g Ei-Phosphatidsäure in 10 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 10 ml 0,01 N Natriumhydroxid-Lösung durch fünf Minuten langes Schütteln bei Raumtemperatur, wobei sich ein pH-Wert von ca. 12 einstellt. Durch Zugabe von 1N Salzsäure wird der pH-Wert der wässrigen Phase auf ca. 7 gebracht. Man erhält eine leicht opaleszierende, wässrige Phase.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop anchgewiesen werden. In der elektronenmikroskopischen Abbildung sind GUL und KUL zu erkennen.

b) Analog Beispiel 8a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure, aber gleicher Menge an Ei-Lecithin und Cholesterin (insgesamt 10 mg Lipid) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den Rückstand in je 1,0 ml einer 0,01 N Natriumhyroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	10	30	50	80
%-KUL	4	10	20	50

Beispiel 9:

Man löst 0,5 g Ei-Phosphatidsäure und 0,5 g Dimyristoyllecithin in 10 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 50 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt 36 %.

Beispiel 10:

Man stellt analog Beispiel 9 Liposomengemische bestehend aus 0,5 g Ei-Phosphatidsäure und jeweils 0,5 g Dipalmitoyllecithin oder Distearoyllecithin her. Die Ausbeute an KUL beträgt 10 %.

Beispiel 11:

Man stellt analog Beispiel 9 ein Liposomengemisch bestehend aus 0,5 g Dipalmitoylphosphatidsäure und 0,5 g Ei-Lecithin her. Die Ausbeute an KUL beträgt 10 %.

Beispiel 12:

Man löst 5 mg Lysolecithin und 5 mg Ei-Lecithin in 1 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser durch fünf Minuten langes Schütteln, wobei sich ein pH-Wert von ca. 5 - 7 einstellt. Zur Bildung von unilamellaren Liposomen gibt man anschliessend zur wässrigen Dispersion bei Raumtemperatur unter Kontrolle mit einem pH-Meter soviel 0,1 N Salzsäure, bis der pH-Wert der wässrigen Phase auf 0,5 abgesunken ist. Durch Zugabe von 0,1 N Natriumhydroxidlösung erhöht man anschliessend den pH-Wert auf 7.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind KUL und GUL zu erkennen. Die Ausbeute an KUL beträgt 50 %.

Beispiel 13:

Man stellt analog Beispiel 12 ein Liposomengemisch bestehend aus 5 mg Phosphatidyl-2-[N,N-dimethyl-N-(2-N',N',N'-trimethylammonioäthyl)-ammonio]-äthylchlorid, dessen Herstellung in Knight C.G., Liposomes, Elsevier 1981, Kapitel 3, beschrieben ist, und 5 mg Ei-Lecithin her.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind KUL mit einem Durchmesser von 250 Å und GUL mit einem Durchmesser von ca. 600 - >10,000 Å zu erkennen. Die Ausbeute an KUL beträgt 50 %.

Beispiel 14:

Man löst 5 mg (6,67 mM) Ei-Lecithin und 5 mg (9,5 mM) natürliches Lysophosphatidylglycerin in 1 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum im Rotationsverdampfer ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser durch fünf Minuten langes Schütteln, wobei sich ein pH-Wert von ca. 5 einstellt. Anschliessend wird der pH-Wert der wässrigen Dispersion mit 0,1 N Natriumhydroxid-Lösung unter Kontrolle mit einem pH-Meter auf ca. 8 eingestellt.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind KUL und GUL zu erkennen. Die Ausbeute an KUL beträgt ca. 35 %.

Beispiel 15:

Man löst 6 mg (8,00 mM) Ei-Lecithin und 4 mg (8,0 mM) natürliches Lysophosphatidylserin in 1 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum im Rotationsverdampfer ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser durch fünf Minuten langes Schütteln, wobei sich ein pH-Wert von ca. 6 einstellt. Anschliessend wird der pH-Wert der wässrigen Dispersion mit 0,1 N-Natriumhydroxid-Lösung unter Kontrolle mit einem pH-Meter auf ca. 8 eingestellt.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind KUL und GUL zu erkennen. Die Ausbeute an KUL beträgt ca. 20 %.

Beispiel 16:

Man löst 5 mg (6,67 mM) Ei-Lecithin und 5 mg (10,0 mM) Lysophosphatidylinositol in 1 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum im Rotationsverdampfer ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser durch fünf Minuten langes Schütteln, wobei sich ein pH-Wert von ca. 6 einstellt. Anschliessend wird der pH-Wert der wässrigen Dispersion mit 0,1 N Natriumhydroxid-Lösung unter Kontrolle mit einem pH-Meter auf ca. 7 eingestellt.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind KUL und GUL zu erkennen. Die Ausbeute an KUL beträgt ca. 40 %.

Beispiel 17:

a) Man löst 0,5 g Monoolein (9-cis-Octadecenoylglycerol) und 0,5 g Ei-Phosphatidsäure in 20 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung im Vakuum ein. Man dispergiert den filmartigen Rückstand in 100 ml einer 0,01 N Natriumhydroxid-Lösung durch fünf Minuten langes Schütteln bei Raumtemperatur, wobei sich ein pH-Wert von ca. 12 einstellt. Durch Zugabe von 1 N Salzsäure wird der pH-Wert der wässrigen Phase auf ca. 7 gebracht. Man erhält eine leicht opalesz ierende, wässrige Phase.

Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind GUL und KUL zu erkennen.

b) Analog Beispiel 17a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure und an Monoolein (totale Lipidmenge = 10 mg) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den Rückstand in je 1 ml einer 0,01 N Natriumhyroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	20	30	50	80
%-KUL	10	17	26	45

Beispiel 18:

Analog Beispiel 17a) löst man Proben unterschiedlichen Gehalts an Ei-Phosphatidsäure und Monomyristin (gesamte Lipidmenge: 10 mg) in je 0,5 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösungen im Vakuum ein. Man dispergiert jeweils den Rückstand in je 1 ml einer 0,01 N Natriumhydroxid-Lösung unter Schütteln, wobei sich ein pH-Wert von ca. 12 einstellt. Der pH-Wert der wässrigen Dispersion wird anschliessend durch Zugabe von 0,1 N Salzsäure auf ca. 7 bis 8 gesenkt. Die Ausbeute an KUL beträgt für jede Probe mit steigendem Ei-Phosphatidsäuregehalt:

%-Ei-Phosphatidsäure	30	50	80
%-KUL	9	18	38

Beispiel 19:

a) Man stellt analog Beispiel 1a) und 1b) ein Liposomengemisch bestehend aus Ei-Phosphatidsäure her, wobei man eine Ausbeute bis zu 66 % KUL erhält. Um den Anteil an GUL im Liposomengemisch zu erhöhen, setzt man zur dispersen Phase, welche frisch hergestellt unilamellare Liposomen enthält, 0,5 molare Kochsalzlösung hinzu. Mit zu-

nehmender NaCl-Konzentration in der dispersen Phase sinkt der Anteil an KUL:

[NaCl] in Mol/l	0	0,2	0,3	0,4	0,5	0,6	0,75	0,95
%-KUL	66	64	50	40	30	23	15	11

b) Um den Anteil an GUL im Liposomengemisch zu erhöhen, setzt man zur dispersen Phase, welche frisch hergestellte unilamellare Liposomen bestehend aus reiner Ei-Phosphatidsäure enthält, 0,5 molare Kaliumchloridlösung hinzu. Mit zunehmender KCl-Konzentration in der dispersen Phase sinkt der Anteil an KUL:

[KCl] in Mol/l	0	0,2	0,4	0,5	0,63
%-KUL	66	63	50	50	36

Beispiel 20:

Man löst 40 mg Dipalmitoylphosphatidylcholin und 20 mg Ei-Phosphatidsäure in 5 ml reinem tert.-Butanol bei 60°. Durch Eintauchen des Kolbens in eine Kältemischung aus Methanol-Trockeneis friert man die Lösung ein. Man entfernt das tert.-Butanol in einem Gefriertrockner und erhält so einen homogenen Schaum von Lipiden. Anschliessend dispergiert man in Wasser durch heftiges Schütteln. Zur Bildung von unilamellaren Liposomen gibt man bei Raumtemperatur unter Kontrolle mit einem pH-Meter soviel 0,1 N-Natronlauge, bis der pH-Wert auf ca. 8 ansteigt. Durch Zugabe von Phosphatpufferlösung stellt man anschliessend den pH-Wert der wässrigen Phase auf ca. 7 ein. Man erhält eine leicht opaleszierende, wässrige Phase. Die gebildeten Liposomen sind im Elektronenmikroskop erkennbar und haben einen Durchmesser von 200 - 10,000 Å.

Beispiel 21:

Man löst 3,0 mg eines in der Tabelle genannten Lipids und 7,0 mg Ei-Lecithin in 1 ml einer Chloroform/Methanol Mischung (2:1) und dampft diese Lösung ein. Man dispergiert den filmartigen Rückstand in 1 ml destilliertem Wasser, wobei sich ein pH-Wert von ca. 6-10 einstellt. Anschliessend erhöht man den pH-Wert der wässrigen Phase durch Zugabe von 0,1 N Natriumhydroxid-Lösung auf ca. 8. Die erfolgte Bildung von unilamellaren Liposomen kann analog Beispiel 1a) spektroskopisch, z.B. im NMR-Spektrum, oder im Elektronenmikroskop nachgewiesen werden. In der elektronenmikroskopischen Abbildung sind GUL und KUL zu erkennen. Die Ausbeute an KUL ist in der Tagelle angegeben:

Tabelle:

Lipid	Konzentration des Lipids [mM/l]	Ausbeute [% KUL]
2-Hydroxyäthyl-3-palmitoyloxyphosphat	6,52	40
2-Methyl-2-palmitoyloxypropylhydrogen-phosphat	6,76	60
3-Cetyloxyprcpyl-2-hydroxyäthylphosphat	6,73	35
2-Bromäthyl-cetylphosphat	6,63	55
n-Eicosyl-2,3-(2,2-propylen)-dioxypropylphosphat	5,84	40
3-Stearyloxypropylhydrogenphosphat	5,98	55
2,3-Dihydroxypropyl-myristylphosphat	7,69	40
3-Cetyloxypropylhydrogenphosphat	7,46	20
2,3-Dihydroxypropyl-n-eicosylphosphat	6,33	7
Cholesteryl-2,3-dihydroxypropyl-phosphat	5,18	70
Cetyl-2,3-dihydroxypropylphosphat	7,18	20
Aethyl-3-stearoyloxypropylphosphat	6,36	40

Beispiel 22:

Analog Beispiel 1-20 kann man unilamellare Liposomen aus Myristinsäure und Ei-Lecithin, Myristinsäure und Ei-Kephalin, Oelsäure und Ei-Lecithin, Oelsäure und Ei-Kephalin, Dimyristoylphosphatidsäure und Dimyristoyllecithin, Dipalmitoylphosphatidsäure und 1-Palmitoyl-2-oleoyllecithin, 1-Palmitoyl-2-oleoylphosphatidsäure und Dipalmitoyllecithin, 1-Palmitoyl-2-oleoylphosphatidsäure und 1-Palmitoyl-2-oleoyllecithin, Ei-Lysophosphatidsäure und Ei-Lecithin, 1-Myristoyllysophosphatidsäure und 1-Palmitoyl-2-oleoyllecithin, 1-Palmitoyllysophosphatidsäure und 1-Palmitoyl-2-oleoyllecithin, Lysophosphatidylserin aus dem Rinderhin und Ei-Lecithin, 1-Palmitoyl-lysophospatidylserin, 1-Palmitoyl-2-oleoyl-phosphatidylserin und 1-Palmitoyl-2-oleoyllecithin und Lysophosphatidylserin aus dem Rinderhirn und Ei-Kephalin herstellen.

Beispiel 23:

2 mg Hydrocortison-21-palmitat, 40 g Ei-Lecithin und 20 mg Ei-phosphatidsäure werden in 5 ml tert.-Butanol gelöst, durch ein 0,2 µm-Filter sterilfiltriert, in eine 25 ml Viale gefüllt, durch Eintauchen der Viale in eine Trockeneis/Aethanol-Kältemischung gefroren und lyophylisiert. Man versetzt den entstandenen Schaum mit 5 ml sterilem destilliertem Wasser und dispergiert durch 10 Minuten langes Schütteln. Durch Zugabe von 0,1 N sterilfiltrierter Natronlauge bringt man den pH-Wert auf 10,5 und lässt eine Minute lang stehen. Anschliessend setzt man 0,5 ml eines 10-fachen Konzentrates von phosphatgepufferter isotonischer Kochsalzlösung von pH 7,4 (PBS für Injektionszwecke) zu. Die so erhaltene Dispersion von unilamellaren Liposomen eignen sich zur Injektion in entzündlich veränderten Gelenkkapseln.

Beispiel 24:

0,1 mg N-Acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoryl)-äthylamid, 7 mg chromatographisch gereinigtes Lecithin aus dem Hühnereiweiss und 3 mg Ei-Phosphatidsäure

werden in 2 ml einer Chloroform/Methanol Mischung (2:1) gelöst und im Vakuum eingedampft. Es bleibt ein klarer Lipidfilm zurück. Man dispergiert diesen Film durch Zugabe von 2 ml sterilem destilliertem Wasser unter Umschwenken und setzt einen Tropfen 0,1 %-iger Thymolphthalein-Lösung hinzu. Zur Dispersion gibt man bis zum Farbumschlag 0,1 N Natronlauge, worauf spontane Bildung von unilamellaren Liposomen erfolgt. Man puffert anschliessend sofort durch Zugabe von 0,2 ml eines 10-fachen Konzentrats von phosphatgepufferter isotonischer Kochsalzlösung (PBS für Injektionszwecke) den pH-Wert auf 7,4 ab.

Die entstandene Dispersion eignen sich direkt zur Aktivierung von alveolären Makrophagen in Zellkulturen oder in vivo in der Ratte.

Beispiel 25:

0,15 g N-Acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoryl)-äthylamid, 27 g Ei-Lecithin mit 97 % Phosphatidylcholingehalt und 3 g Ei-Phosphatidsäure werden in einer Mischung von 200 ml Chloroform und 20 ml Methanol gelöst, mit 200 ml tert.-Butanol aufgefüllt und auf 180 ml eingeengt. Die Lösung wird mit einem 0,2 µm Filter sterilfiltriert, in einer Aethanol/Trockeneis Mischung rasch gefroren und anschliessend gefriergetrocknet. Das durch Mahlung zerkleinerte Lyophilisat wird in 300 ml steril hergestellte 0,01 N Natriumhydroxid-Lösung unter kräftigem Rühren eingetragen. Nach vollständiger Dispersion wird die wässrige Phase durch Zugabe von 0,1 n HCl neutralisiert. Die erhaltene opaleszierende Dispersion wird in eine gerührte Ultrafiltrationszelle (Amicon®), eingefüllt, die anstelle des Ultrafilters mit einem geradporigen Filter aus Polcarbonat (Nucelopore®) mit einem Porendurchmesser von 0,1 µm versehen ist und partikelfrei gewaschen wurde, und unter geringem Ueberdruck und stetiger Zufuhr von sterilfiltrierter Pufferlösung nach Dulbecco (pH 7,4 ohne Ca und Mg) so filtriert, dass das Volumen in der Zelle nicht unter 300 ml sinkt. Nach Durchtritt von 3 l Filtrat sind alle GUL abgetrennt und die überstehende Dispersion an GUL kann ampulliert und für Behandlungsversuche eingesetzt werden.

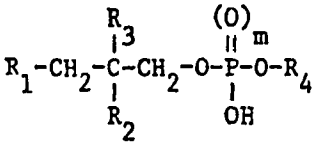
Beispiel 26:

15 mg N-Acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2'-di-palmitoyl-sn-glycero-3'-phosphoryl)-äthylamid, 0,6 g reines Ei-Lecithin und 2,4 g Ei-Phosphatidsäure werden in einer Mischung von 20 ml Chloroform und 2 ml Methanol gelöst, durch ein 0,2 µm-Filter sterilfiltriert und an einem partikelfrei gewaschenen, über Sterilfilter entlüfteten Rotationsverdampfer in einem 500 ml Rundkolben so eingedampft, dass die Lipidmischung als möglichst gleichmässiger Film auf der Kolbenwand trocknet. Nach Trocknung des Rückstands über Nacht im Hochvakuum werden 30 ml steril hergestellte 0,01 N Natriumhydroxid-Lösung zugegeben, der Kolben verschlossen und 5 Minuten lang geschüttelt. Die entstandene opaleszierende wässrige Phase wird durch Zugabe von steriler 0,1 N Salzsäure auf pH 7,4 gestellt. Nach Einfüllen in eine gerührte Filterzelle (Totalvolumen 100 ml) gemäss Beispiel 23 wird unter Zugabe von sterilem, partikelfrei filtriertem Wasser so lange filtriert, bis 500 ml Filtrat gesammelt sind. Dieses Filtrat wird in eine gerührte Filterzelle, die mit einem Ultrafilter, z.B. Amicon U 10®, bestückt ist, kontinuierlich eingespeist und auf ein Volumen von 30 ml konzentriert. Die konzentrierte Dispersion enthält kleine, unilamellare Liposomen und kann nach Zugabe eines Konzentrats von Phosphatpuffer nach Dulbecco (pH 7,4 ohne Ca und Mg) ampulliert und für Behandlungsversuche eingesetzt werden.

Ansprüche

1. Verfahren zur Herstellung von unilamellaren Liposomen, dadurch gekennzeichnet, dass man

a) ein Lipid der Formel



(A),

worin m null oder ein ist, einer der Reste R_1 und R_2 Wasserstoff, Hydroxy, Niederalkyl mit 1-4 C-Atomen und der andere Rest Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff oder Niederalkyl mit 1-4 C-Atomen und R_4 Wasserstoff, Niederalkyl mit 1-7 C-Atomen, einen Kohlehydratrest mit 5-12 C-Atomen oder, wenn R_1 und R_2 Wasserstoff oder Hydroxy und R_3 Wasserstoff bedeuten, einen Steroidrest bedeuten, und ein geeignetes zusätzliches Lipid und/oder eine Fettsäure und ein geeignetes zusätzliches Lipid mit Ausnahme eines Sterins oder

ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 und R_4 Wasserstoff bedeuten und gegebenenfalls ein geeignetes zusätzliches Lipid in wässriger Phase mit einem pH-Wert grösser als 7 dispergiert, oder

b) ein Lipid der Formel A, worin m null oder eins ist, einer der Reste R_1 und R_2 Wasserstoff, Hydroxy, Niederalkyl mit 1-4 C-Atomen und der andere Rest Alkyl, Alkenyl, Alkoxy oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammoniogruppe substituiertes Niederalkyl bedeuten, und gegebenenfalls ein geeignetes zusätzliches Lipid oder

ein Lipid der Formel A, worin m null oder eins ist, R_1 und R_2 unabhängig voneinander Alkyl, Alkenyl oder Alkenyloxy mit je 10-20 C-Atomen oder Acyloxy mit 10-50 C-Atomen, R_3 Wasserstoff und R_4 durch eine Ammonioniederalkylammoniogruppe substituiertes Niederalkyl bedeuten, und ein geeignetes zusätzliches Lipid in wässriger Phase mit einem pH-Wert kleiner als 7 dispergiert, und, wenn notwendig, die wässrige Phase neutralisiert und, wenn erwünscht, die erhältlichen unilamellaren Liposomen anreichert und/oder abtrennt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion ein Lipid der Formel A, worin m eins ist, R_1 Alkyl, z.B. n-Dodecyl (Lauryl), n-Tridecyl, n-Tetradecyl (Myristyl), n-Pentacedyl, n-Hexadecyl (Cetyl), n-Heptadecyl oder n-Octadecyl (Stearyl), Alkoxy, z.B. n-Dodecyloxy (Lauryloxy), n-Tetradecyloxy (Myristyloxy), n-Hexadecyloxy (Cetyloxy), oder n-Octadecyloxy (Stearyloxy), Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_2 Wasserstoff oder Hydroxy, R_3 Wasserstoff oder Niederalkyl, z.B. Methyl, und R_4 Wasserstoff, Niederalkyl, z.B. Methyl oder Aethyl, Niederalkyl substituiert durch saure und basische Gruppen, z.B. Carboxy und Amino, z.B. ω-Amino-ω-carboxyniederalkyl, z.B. 2-Amino-2-carboxyäthyl oder 3-Amino-3-carboxy-n-propyl, Hydroxyniederalkyl, z.B. 2-Hydroxyäthyl oder 2,3-Hydroxypropyl, Niederalkylendioxyniederalkyl, z.B. 2,3-Aethylendioxypropyl oder 2,3-(2,2-Propylen)-dioxypropyl, Halogenniederalkyl, z.B. 2-Chlor- oder 2-Bromäthyl, einen Kohlehydratrest mit 5-12 C-Atomen, z.B. Inosit, oder einen Steroidrest, z.B. ein Sterin, z.B. Cholesterin bedeuten, und ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl oder 2-Aminoäthyl bedeuten, enthält.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Disperison ein Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy,

R_3 Wasserstoff und R_4 Wasserstoff bedeuten, und gegebenenfalls ein zusätzliches Lipid der Formel A, worin R_1 und R_2 Acyloxy, z.B. Lauroyloxy, Myristoyloxy, Palmitoyloxy oder Stearoyloxy, R_3 Wasserstoff und R_4 2-Trimethylammonioäthyl, 2-Aminoäthyl, Niederalkyl substituiert durch saure und basische Gruppen, z.B. Carboxy und Amino, z.B. ω-Amino-ω-carboxyniederalkyl, z.B. 2-Amino-2-carboxyäthyl oder 3-Amino-3-carboxy-n-propyl, oder einen Kohlehydratrest mit 5-12 C-Atomen bedeuten, z.B. Inosil, oder ein Monoglycerid, z.B. Monoolein oder Monomyristin, oder ein Stearin, z.B. Cholesterin, enthält.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion eine Lysophospahtidsäure, z.B. eine natürliche Lysophosphatidsäure, z.B. Ei-Lysophosphatidsäure, oder eine synthetische Lysophosphatidsäure, z.B. 1-Lauroyl-, 1-Myristoyl- oder 1-Palmitoyllysophosphatidsäure, ein Lysophosphatidylserin, z.B. ein natürliches Lysophosphatidylserin, z.B. Lysophosphatidylserin aus dem Rinderhirn, oder ein synthetisches Lysophosphatidylserin, z.B. 1-Myristoyl- oder 1-Palmitoyllysophosphatidylserin, ein Lysophosphatidylglycerin oder ein Lysophosphatidylinositol und zusätzlich ein Lecithin, z.B. ein natürliches Lecithin, z.B. Ei-Lecithin, oder ein Lecithin mit gleichen Acyloxygruppen, z.B. Dimyristoyl- oder Dipalmitoyllecithin, ein Lecithin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoyllecithin, oder zusätzlich ein Kephalin, z.B. ein natürliches Kephalin, z.B. Ei-Kephalin, oder ein Kephalin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoylkephalin.

5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion eine natürliche Phosphatidsäure, z.B. Ei-Phosphatidsäure, eine synthetische Phosphatidsäure, z.B. Dilauroyl-, Dimyristoyl-, Dipalmitoyl- oder 1-Palmitoyl-2-oleoylphosphatidsäure, und gegebenenfalls zusätzlich ein Lecithin, z.B. ein natürliches Lecithin, z.B. Ei-Lecithin, ein Lecithin mit gleichen Acyloxygruppen, z.B. Dimyristoyl- oder Dipalmitoyllecithin, oder ein Lecithin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoyllecithin, oder ein Kephalin, z.B. ein natürliches Kephalin, z.B.

Ei-Kephalin oder ein Kephalin mit verschiedenen Acyloxygruppen, z.B. 1-Palmitoyl-2-oleoylkephalin, oder ein Phosphatidylserin, z.B. ein natürliches Phosphatidylserin, z.B. Phosphatidylserin aus dem Rinderhirn, oder ein synthetisches Phosphatidylserin, z.B. Dipalmitoylphosphatidylserin, ein Monoglycerid, z.B. Monoolein oder Monomyristin, oder ein Sterin, z.B. Cholesterin, enthält.

6. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion Ei-Phosphatidsäure oder Ei-Phosphatidsäure und Ei-Lecithin enthält.

7. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion Ei-Phosphatidsäure, Ei-Lecithin oder Phosphatidylserin aus dem Rinderhirn enthält.

8. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Disperison Asolectin enthält.

9. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion Ei-Phosphatidsäure, Ei-Lecithin und Cholesterin enthält.

10. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion Lysolecithin und Ei-Lecithin enthält.

11. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass die wässrige Dispersion natürliches Lysophosphatidylserin und Ei-Lecithin enthält.

12. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man eine homogene Schicht der unter Verfahren a) genannten Lipide in wässriger Phase dispergiert und anschliessend den pH-Wert bis auf ca. 12 erhöht.

13. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass man den pH-Wert durch Zugabe von verdünnter wässriger Natriumhydroxid- oder Kaliumhydroxid-Lösung erhöht.

14. Verfahren nach Anspruch 12, dadurch gekennzeichnet, dass man eine homogene Schicht der unter Verfahren a) genannten Lipide in wässrigen Phasen mit einem pH-Wert grösser als 7 dispergiert.

15. Verfahren nach Anspruch 14, dadurch gekennzeichnet, dass man eine homogene Schicht der unter Verfahren a) genannten Lipide in verdünnter, wässriger Natriumhydroxid- oder Kaliumhydroxid-Lösung dispergiert.

16. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man eine homogene Schicht der unter Verfahren b) genannten Lipide in wässriger Phase mit einem pH von ca. 1 dispergiert.

17. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man die wässrige Phase anschliessend durch Zugabe von physiologisch annehmbaren Säuren, Basen oder Pufferlösung mit einem pH 7-8 neutralisiert.

18. Verfahren nach Anspruch 17, dadurch gekennzeichnet, dass man eine solche wässrige Phase durch Zugabe von physiologisch annehmbaren Säuren oder Pufferlösung mit einem pH 7-8 neutralisiert, die man zuvor auf Werte höher als pH 8 eingestellt hat.

19. Verfahren nach Anspruch 18, dadurch gekennzeichnet, dass man die wässrige Phase durch Zugabe von Salzsäure neutralisiert.

20. Verfahren nach Anspruch 1, dadurch gekennzeichnet, dass man eine solche wässrige Phase durch Zugabe von physioloigsch annehmbaren Basen neutralisiert, die man zuvor auf Werte niedriger als pH 5 eingestellt hat.

21. Die nach dem Verfahren gemäss Anspruch 1 erhältlichen unilamellaren Liposomen.

22. Verabreichungssystem auf Liposomenbasis für verkapselte Wirkstoffe, hergestellt nach dem Verfahren gemäss Patentanspruch 1.

23. Verabreichungssystem auf Liposomenbasis für verkapseltes N-Acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2'-dipalmitoyl-sn-glycero-3'-phosphoro)-äthylamid, hergestellt nach den Verfahren gemäss Patentanspruch 1.

24. Pharmazeutische Zusammensetzung enthaltend ein Verabreichungssystem auf Liposomenbasis für verkapselte Wirkstoffe gemäss Anspruch 18, vermischt mit pharmazeutisch verträglichen Zusatzstoffen.

25. Verabreichungssystem gemäss Anspruch 22 zur Anwendung bei der Behandlung des menschlichen oder tierischen Körpers.

26. Pharmazeutische Zusammensetzung gemäss Anspruch 22 zur Anwendung bei der Behandlung des menschlichen oder tierischen Körpers.

27. Die Methode der Behandlung von Erkrankungen des menschlichen oder tierischen Körpers mit Verabreichungssystemen gemäss Anspruch 22.

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(54) AQUEOUS SUSPENSION PREPARATIONS WITH EXCELLENT REDISPERSIBILITY

(57) The aqueous suspension can be prepared by incorporating, in an aqueous suspension of a hardly soluble drug, a water-soluble polymer within the concentration range from the concentration at which the surface tension of the aqueous suspension of the drug begins to decrease up to the concentration at which the reduction in surface tension ceases. The resulting aqueous suspension shows ready redispersibility and will not undergo aggregation of dispersed particles or caking. Because of its good redispersibility, the suspension is useful as a parenteral preparation, eye drops, nasal drops, a preparation for oral administration, a lotion or the like.

EP 0 995 435 A1

Description

Technical Field

[0001] The present invention relates to an aqueous suspension with good redispersibility.

Background Art

[0002] In preparing, for instance, an ophthalmic preparation, nasal preparation or parenteral preparation containing a medicinal compound hardly soluble in water, it is presumable that the drug be suspended in an aqueous medium to give an aqueous suspension. When such aqueous suspension is stored for a long period, the drug occurring as dispersed particles (hereinafter sometimes referred to merely as dispersed particles) tends to undergo aggregation, resulting in increases in size of dispersed particles or sedimentation of dispersed particles and further in secondary aggregation of the dispersed particles that have settled, for example caking. Therefore, efforts have been devoted to prevent the aggregation or sedimentation of dispersed particles as far as possible or, when such aggregation or sedimentation cannot be prevented, to obtain suspensions capable of readily regaining their original state.

[0003] One method so far proposed comprises making dispersed particles smaller, decreasing the difference between the specific gravity of dispersed particles with of the dispersion medium and increasing the viscosity of the dispersion medium to thereby prevent the particles from settling. In such cases, for increasing the viscosity of the dispersion medium, the concentration of the suspending agent and/or thickening agent, such as a water-soluble polymer, has generally been selected within the range of 0.2 to 5.0% (w/v: weight/volume).

[0004] However, even when the concentration of the suspending agent and/or thickening agent is within such range, the sedimentation of particles cannot entirely be prevented. The problem which remains is that dispersed particles settle and deposit, causing caking, resulting in failure of uniform redispersion.

[0005] Another method which is conceivable comprises making drug particles greater in size to thereby improve their redispersibility. In the case of an ophthalmic preparation, however, greater particle sizes may cause a foreign matter sensation or eye irritation upon instillation. In the case of a nasal preparation, greater particle sizes make it impossible to apply it from a spray bottle. In the case of an injection, it is a drawback that it cannot be administered through a needle.

Disclosure of Invention

[0006] Among the drugs recently developed and producing pharmacological effects of value, many are hardly soluble ones. For supplying these in the form of aqueous preparations such as ophthalmic, nasal, parenteral and other preparations, it is unavoidable in many instances to employ the aqueous suspension form. However, the prior art aqueous suspensions have a redispersibility problem; in many instances, it is difficult to restore suspensions uniform in concentration without a long time of shaking to effect redispersion. Thus, the advent of aqueous drug suspensions which can be readily prepared and have good redispersibility has been waited for. Accordingly, it is the primary object of the present invention to provide an aqueous suspension showing good redispersibility without undergoing aggregation of dispersed particles or caking.

[0007] The present inventors made intensive investigations to solve the above problems and, as a result, found that there is a certain relationship between the surface tension of an aqueous suspension and the redispersibility thereof. Based on such finding, they have now completed the present invention.

[0008] The invention is thus concerned with an aqueous suspension comprising a hardly soluble drug together with a water-soluble polymer within the concentration range from the concentration at which the surface tension of the drug suspension begins to decrease up to the concentration at which the reduction in surface tension ceases.

[0009] As will be shown later herein in Test Example 1, the surface tension of an aqueous suspension begins to decrease with the increase in the amount of a water-soluble polymer added thereto. Upon continuation of the addition, the reduction in surface tension ceases and, thereafter, a substantially constant surface tension is maintained. On the contrary, the redispersibility of the dispersed particles of an aqueous suspension becomes good at the point at which the surface tension of the aqueous suspension begins to decrease as a result of addition of the water-soluble polymer, and the good redispersibility is maintained until the reduction in surface tension ceases. Thereafter, as the reduction in surface tension ceases and the surface tension becomes constant, the redispersibility of the dispersed particles becomes gradually worsened.

[0010] Where no water-soluble polymer is present, the dispersed particles aggregate together and float on the surface of the suspension and therefore no uniform suspension can be prepared.

[0011] The concentration of a water-soluble polymer at which the surface tension of an aqueous drug suspension begins to decrease and the concentration of the water-soluble polymer at which the reduction in surface tension ceases

generally increase according to the contents of the hardly soluble drug used in the aqueous suspension but vary depending on the physical properties, chemical structure, and concentration and particle size of the hardly soluble drug, among others. The water-soluble polymer concentration at which the surface tension of the drug suspension begins to decrease is generally 0.00001 to 0.01% (w/v), preferably 0.00005 to 0.005% (w/v), while the water-soluble polymer concentration at which the reduction in surface tension of the suspension ceases is generally 0.0001 to 0.1% (w/v), preferably 0.001 to 0.01% (w/v).

[0012] The aqueous suspension of the present invention is generally prepared at a water-soluble polymer concentration within the range of 0.00001 to 0.1% (w/v), preferably 0.00005 to 0.05% (w/v), more preferably 0.0001 to 0.01 w/v %.

[0013] The ratio of the water-soluble polymer to the hardly soluble drug is generally 0.0001 to 0.2 part by weight, preferably 0.0005 to 0.1 part by weight, more preferably 0.0005 to 0.05 part by weight of the former to 1 part by weight of the latter.

[0014] The water-soluble polymer to be used in the practice of the present invention may be any pharmaceutically acceptable water-soluble polymer, irrespective of type or category. Cellulose derivatives and water-soluble polyvinyl polymers are suited for use, however.

[0015] As the cellulose derivatives, there may be mentioned, for example, hydroxypropylmethylcellulose, methylcellulose, hydroxyethylcellulose and hydroxypropylcellulose. Particularly preferred among them are hydroxypropylmethylcellulose and methylcellulose.

[0016] As the water-soluble polyvinyl polymers, there may be mentioned, among others, polyvinylpyrrolidone K25, polyvinylpyrrolidone K30, polyvinylpyrrolidone K90, and polyvinyl alcohol (partial hydrolyzed product, complete hydrolyzed product).

[0017] As used herein, the "hardly soluble drug" includes, within the meaning thereof, those drugs which belong, in solubility classification, to one of the groups "sparingly soluble", "slightly soluble", "very slightly soluble" and "practically insoluble" as so defined in the Japanese Pharmacopoeia. Thus, it includes all drugs that can be provided in the final dosage form of aqueous suspensions.

[0018] As specific examples of the hardly soluble drug to be used in the practice of the present invention, there may be mentioned steroidal antiinflammatory agents, antiinflammatory analgesics, chemotherapeutic agents, synthetic antibacterial agents, antiviral agents, hormones, anticataract agents, neovascularization inhibitors, immunosuppressants, protease inhibitors, and aldose reductase inhibitors, among others. The steroidal antiinflammatory agents include, among others, cortisone acetate, hydrocortisone acetate, betamethasone, prednisolone, fluticasone propionate, dexamethasone, triamcinolone, loteprednol, fluorometholone, difluprednate, momethasone furoate, clobetasol propionate, diflorasone diacetate, diflucortolone valerate, fluocinonide, amcinonide, halcinonide, fluocinolone acetonide, triamcinolone acetonide, flumetasone pivalate and clobetasone butyrate. The antiinflammatory analgesics include, among others, alclofenac, aluminopropfen, ibuprofen, indomethacin, epirizole, oxaprozin, ketoprofen, diclofenac sodium, diflunisal, naproxen, piroxicam, fenbufen, flufenamic acid, flurbiprofen, floctafenine, pentazocine, metiazinic acid, mefenamic acid and mofezolac. The chemotherapeutic agents include, among others, sulfa drugs such as salazusulfapyridine, sulfadimethoxine, sulfamethizole, sulfamethoxazole, sulfamethopyrazine and sulfamonomethoxine, synthetic antibacterial agents such as enoxacin, ofloxacin, cinoxacin, sparfloxacin, thiamphenicol, nalidixic acid, tosufloxacin tosilate, norfloxacin, pipemidic acid trihydrate, piromidic acid, fleroxacin and levofloxacin, antiviral agents such as aciclovir, ganciclovir, didanosine, didovudine and vidarabine, and antifungal agents such as itraconazole, ketoconazole, fluconazole, flucytosine, miconazole and pimaricin. The hormones include, among others, insulin zinc, testosterone propionate and estradiol benzoate. The anticataract agents include, among others, pirenoxine and the like. The neovascularization inhibitors include, among others, fumagillin and derivatives thereof. The immunosuppressants include, among others, ciclosporin, rapamycin and tacrolimus. The protease inhibitors include, among others, [L-3-trans-ethoxycarbonyloxiran-2-carbonyl]-L-leucine (3-methylbutyl)amide (E-64-d) and the like. The aldose reductase inhibitors include, among others, 5-(3-ethoxy-4-pentyloxyphenyl)thiazolidine-2,4-dione and the like.

[0019] The concentration of the hardly soluble drug to be used in the practice of the invention may vary according to the drug species, indication, dosage and other factors. Generally, however, it is 0.01 to 10.0% (w/v), preferably 0.1 to 5.0% (w/v).

[0020] The aqueous suspension of the present invention may contain, in addition to the hardly soluble drug and water-soluble polymer, known compounds such as a buffer (e.g. carbonate salt, phosphate salt, acetate salt, glutamic acid, citrate salt, ε -aminocaproic acid), an isotonizing agent (e.g. glycerol, mannitol, sorbitol, propylene glycol, sodium chloride, potassium chloride, boric acid), a stabilizer (e.g. sodium edetate, sodium citrate), a surfactant (e.g. polysorbate 80, polyoxyethylene(60) hydrogenated castor oil, tyloxapol, benzalkonium chloride), a preservative (p-hydroxybenzoate and it's analogs, benzalkonium chloride, benzethonium chloride, chlorobutanol), a pH control agent (e.g. hydrochloric acid, sodium hydroxide, phosphoric acid), and other additives.

[0021] In cases where an additive which may influence the surface tension of the aqueous suspension, for example a surfactant, is used, it is preferred that the surface tension measurement be made prior to addition of the surfactant

and the surfactant be added after selection of the concentration of the water-soluble polymer.

[0022] The pH of the aqueous suspension of the present invention is not critical but, generally, it is 4 to 9, preferably 5 to 8. It is preferred that the surface tension be selected according to the intended pH of the aqueous suspension.

[0023] The aqueous suspension of the invention has good redispersibility without involving aggregation or caking of dispersed particles and, therefore, can be used with advantage as, for example, an ophthalmic preparation, a preparation for nasal application, an injection, a preparation for oral administration or a lotion.

Brief Description of Drawings

[0024]

Fig. 1 is a graphic representation of the relationships between the HPMC concentration and the surface tension and redispersion time for a 0.1% (w/v) fluorometholone suspension as found in Test Example 1. In the figure, -●-indicates the surface tension, and -♦- indicates the redispersion time.

Best Mode for Carrying Out the Invention

[0025] The following test examples and working examples illustrate the invention in further detail. They are, however, by no means limitative of the scope of the invention.

Test Example 1 Surface tension-redispersibility test

[Method]

[0026] Solutions containing a suspending agent in concentrations ranging from 0.000001 to 0.5 w/v % were first prepared. A test drug was added to the solutions to prepare aqueous suspensions. The surface tension of each aqueous suspension thus prepared was measured with the Du Noüy tension meter K122 (Krüss). The suspensions were then filled into 5 ml colorless polypropylene bottles and allowed to stand at 25°C for 4 days. Each bottle was caused to spin (60 rpm) on the variable mix rotor VMR-5 (60 rpm, manufactured by Iuchi) and the time required for redispersion was measured. In addition, the condition of the redispersed particles was visually examined.

[0027] As the suspending agent, hydroxypropylmethyl cellulose [Metolose 60SH (TC-5E); manufactured by Shin-Etsu Chemical Co., Ltd.; hereinafter abbreviated as HPMC], methylcellulose (Metolose SM-25; manufactured by Shin-Etsu Chemical Co., Ltd.; abbreviated as MC), or polyvinylpyrrolidone (K30; manufactured by BASF; abbreviated as PVP) was used. As the test drug, fluorometholone 0.05 w/v % or 0.1 v/v % or indomethacin 0.2 w/v % or 1.0 w/v % was used.

[Results]

(1) Relationship of the concentration of HPMC to the surface tension and redispersion time of fluorometholone 0.1 w/v % suspension

[0028] The relation between the surface tension and redispersibility of a fluorometholone 0.1 w/v % suspension is shown in Fig. 1.

[0029] In the case of HPMC, the surface tension began to decline at 0.0001 w/v % and the decrease in surface tension almost ceased at 0.01 w/v. On the other hand, within the concentration range of 0.000005 to 0.0001 w/v % HPMC, the time required for redispersion was 2 seconds but the dispersed particles aggregated and floated, failing to give a uniform suspension. Over the range of 0.0001 to 0.01 w/v % HPMC, the redispersion time was less than 4 seconds and a uniform suspension was obtained without aggregation of suspended particles. When the concentration of HPMC was over 0.01 w/v %, the redispersion time exceeded 5 seconds, indicating that the redispersibility is adversely affected.

[0030] The preferred ratio of HPMC to fluorometholone was found to be 0.001 to 0.1 part by weight of the former to 1 part by weight of the latter.

(2) Relationship of the concentration of HPMC to the surface tension of fluorometholone 0.05 w/v % suspension

[0031] With HPMC, the surface tension began to decline at 0.0001 w/v % (surface tension: 65.1 mN/m) and the decrease in surface tension almost ceased at 0.002 w/v % HPMC (surface tension: 50.5 mN/m). The time required for redispersion of fluorometholone in this concentration range of HPMC was about 6 seconds and the condition of the dispersion was satisfactory.

[0032] The preferred ratio of HPMC to fluorometholone was 0.002 to 0.04 part by weight of the former to 1 part by weight to the latter.

(3) Relationship of the concentration of MC to the surface tension and redispersion time of fluorometholone 0.1 w/v % suspension

[0033] With the concentration of MC being 0.0001 w/v % and below, the surface tension was almost constant at 72.5 mN/m. The surface tension began to decline at 0.0001 w/v % MC and the decrease in surface tension almost ceased at 0.01 w/v %, when a tension value of 54.5 mN/m was recorded. On the other hand, when the concentration of MC was 0.0001 w/v % or less, the redispersion time was as short as 2 seconds or less but the dispersed particles aggregated and floated, failing to give a uniform suspension. Within the concentration range of 0.0001 to 0.01 w/v % MC, the necessary redispersion time was 9 to 10.7 seconds, with redispersion taking place rapidly without aggregation of dispersed particles. When the concentration of MC was over 0.01 w/v %, the redispersion time was found to be close to 20 seconds, with redispersibility being adversely affected.

[0034] The preferred ratio of MC to fluorometholone was 0.001 to 0.1 part by weight of the former to 1 part by weight of the latter.

(4) Relationship of the concentration of HPMC to the surface tension and redispersion time of indomethacin 0.2 w/v % suspension

[0035] When the concentration of HPMC was less than 0.0001 w/v %, the surface tension was almost constant at 72 mN/m. The surface tension began to decline at 0.0001 w/v % HPMC and the decrease in surface tension almost ceased at 0.01 w/v % HPMC, with a tension value of 48 mN/m being recorded. On the other hand, when the concentration of HPMC was below 0.0001 w/v %, the redispersion time was as short as 7 seconds or less but the dispersed particles aggregated and floated, failing to give a uniform suspension. Within the concentration range of 0.0001 to 0.01 w/v % HPMC, the redispersion time was 6.3 to 8.3 seconds, with the drug being rapidly redispersed without aggregation. When the concentration of HPMC was over 0.01 w/v %, the redispersibility was found to deteriorate, with the redispersion time exceeding 12 seconds.

[0036] The preferred ratio of HPMC to indomethacin was 0.0005 to 0.05 part by weight of the former to 1 part by weight of the latter.

(5) Relationship of the concentration of HPMC to the surface tension and redispersion time of indomethacin 1.0 w/v % suspension

[0037] When the concentration of HPMC was below 0.0005 w/v %, the surface tension was almost constant at 72.73 mN/m. The surface tension began to decline at 0.0005 w/v % HPMC and the decrease in surface tension almost ceased at 0.005 w/v %, at which level a tension value of 49.7 mN/m was recorded. On the other hand, when the concentration of HPMC was less than 0.0005 w/v %, the redispersion time was not more than 7 seconds but the dispersed particles aggregated and floated, failing to give a uniform suspension. Within the concentration range of 0.0005 to 0.005 w/v % HPMC, the redispersion time was 7.3 to 16 seconds, with the drug particles being rapidly redispersed without aggregation. When the concentration of HPMC exceeded 0.005 w/v %, the redispersion time was increased to more than 20 seconds, with the redispersibility deteriorating.

[0038] The preferred ratio of HPMC to indomethacin was 0.0005 to 0.005 part by weight of the former to 1 part by weight of the latter.

(6) Relationship of the concentration of PVP to the surface tension of fluorometholone 0.05 w/v % suspension

[0039] The surface tension began to decline at 0.0002 w/v % PVC (surface tension: 72.3 mN/m) and the decrease in surface tension almost ceased at 0.001 w/v % (surface tension: 69.5 mN/m).

[0040] The preferred ratio of PVP to fluorometholone was 0.004 to 0.02 part by weight of the former to 1 part by weight of the latter.

(7) Relationship of the concentration of PVP to the surface tension of fluorometholone 0.1 w/v % suspension

[0041] When the concentration of PVP was less than 0.0003 w/v %, the surface tension was almost constant at 72.5 mN/m. The surface tension began to decline at 0.0003 w/v % and the decrease in surface tension almost ceased at 0.002 w/v % where a tension value of 69.5 mN/m was recorded. The time necessary for redispersion of fluorometholone in this concentration range was about 6 seconds and the condition of the dispersion was satisfactory. When the

concentration of PVP was in excess of 0.002 w/v %, the redispersion time was prolonged to 18 seconds or longer, with the redispersibility being found to deteriorate.

[0042] The preferred ratio of PVP to indomethacin was 0.003 to 0.02 part by weight of the former to 1 part by weight of the latter.

[0043] The above results indicate that although the surface tension of the aqueous suspension is dependent on the kind of water-soluble polymer added and the kind and concentration of hardly soluble drug, suspensions of hardly soluble drugs with good redispersibility can be prepared within the concentration range of the water-soluble polymer from the level where the surface tension begins to decline to the level where the decrease in surface tension ceases, regardless of the kind of water-soluble polymer.

Test Example 2

Redispersibility test under accelerated conditions

[Method]

[0044] Ophthalmic preparations were prepared according to Examples 2 and 4 presented below and each preparation was filled in a 5 ml polypropylene bottle. After the bottle was centrifuged at 200 G for 10 minutes for forced sedimentation of the suspended particles, it was caused to spin (60 rpm) on the variable mix rotor VMR-5 (60 rpm, Iuchi) and the redispersion time was measured.

[Results]

[0045] The redispersion times of the ophthalmic preparations of Examples 2 and 4 were 4 seconds and 7 seconds, respectively. Gross observation of each redispersed suspension showed a uniform dispersion of fine particles.

[0046] The above results indicate that in the case of the aqueous suspension according to the invention, its redispersibility is well maintained even under the rugged condition of forced sedimentation of the particles by centrifugation and is not affected by the buffer and preservative ingredients, either.

Example 1: Ophthalmic preparation

[0047]

Fluorometholone	0.1 g
Methylcellulose	0.0006 g
Sodium chloride	0.85 g
Disodium hydrogenphosphate dodecahydrate	0.1 g
Benzalkonium chloride	0.005 g
0.1 N Hydrochloric acid	q.s. to make pH 7.0
Purified water	q.s. to make 100 ml.

[0048] Methylcellulose was dissolved in about 80 ml of purified water by effecting dispersion with warming, followed by cooling to room temperature. Sodium chloride, Disodium hydrogenphosphate dodecahydrate and benzalkonium chloride were added for dissolution. The pH was adjusted to 7 by adding hydrochloric acid. Fluorometholone was added and uniform suspension was effected using a homogenizer. Purified water was added to make the whole volume 100 ml. A fluorometholone suspension ophthalmic preparation was thus prepared.

Example 2: Ophthalmic preparation

[0049]

Fluorometholone	0.05 g
Methylcellulose	0.0003 g
Sodium chloride	0.85 g
Sodium dihydrogen phosphate dihydrate	0.1 g
Benzalkonium chloride	0.005 g
0.1 N Sodium hydroxide	q.s. to make pH 7.0
Purified water	q.s. to make 100 ml.

[0050] A fluorometholone suspension ophthalmic preparation was prepared in the same manner as in Example 1.

Example 3: Ophthalmic preparation

[0051]

Fluorometholone	0.02 g
Methylcellulose	0.0001 g
Sodium chloride	0.85 g
Disodium hydrogenphosphate dodecahydrate	0.1 g
Benzalkonium chloride	0.005 g
0.1 N Hydrochloric acid	q.s. to make pH 7.0
Purified water	q.s. to make 100 ml.

[0052] A fluorometholone suspension ophthalmic preparation was prepared in the same manner as in Example 1.

Example 4: Ophthalmic preparation

[0053]

Fluorometholone	0.05 g
Polyvinylpyrrolidone K30	0.0015 g
Sodium chloride	0.9 g
Sodium dihydrogen phosphate dihydrate	0.1 g
Benzalkonium chloride	0.005 g
0.1 N Sodium hydroxide	q.s. to make pH 7.0
Purified water	q.s. to make 100 ml.

[0054] Polyvinylpyrrolidone, sodium chloride, sodium dihydrogen phosphate dihydrate and benzalkonium chloride were added to about 80 ml of purified water and dissolution was effected. The pH was adjusted to 7 by adding 0.1N sodium hydroxide. Fluorometholone was added and uniform suspension was effected ultrasonically. The whole volume was made 100 ml by adding purified water. A fluorometholone suspension ophthalmic preparation was thus prepared.

Example 5: Ophthalmic preparation

[0055]

Sulfamonomethoxine	0.1 g
Hydroxypropylmethylcellulose	0.001 g
Sodium acetate	0.1 g
Benzalkonium chloride	0.005 g
Sodium chloride	0.9 g
0.1 N Hydrochloric acid	q.s. to make pH 5.0
Purified water	q.s. to make 100 ml.

[0056] Hydroxypropylmethylcellulose was dissolved in about 80 ml of purified water by effecting dispersion with warming, followed by cooling to room temperature. Sodium chloride, sodium acetate and benzalkonium chloride were added and dissolution was effected. The pH was adjusted to 5 by adding hydrochloric acid. Sulfamonomethoxine was added and uniform suspension was effected by means of a mill. The whole amount was made 100 ml by adding purified water. A sulfamonomethoxine suspension ophthalmic preparation was thus prepared.

Example 6: Nasal drops

[0057]

Hydrocortisone acetate	0.1 g
Hydroxypropylmethylcellulose	0.0008 g
Sodium dihydrogen phosphate	0.1 g
Methylparaben	0.026 g
Propylparaben	0.014 g
Concentrated glycerin	2.6 g
0.1 N Sodium hydroxide	q.s. to make pH 7.0
Purified water	q.s. to make 100 ml.

[0058] Methylparaben and propylparaben were dissolved in about 80 ml of purified water with warming. Hydroxypropylmethylcellulose was dispersed in the warm solution for effecting dissolution, followed by cooling to room temperature. Concentrated glycerin and sodium dihydrogen phosphate were added and dissolution was effected. The pH was adjusted to 7 by adding sodium hydroxide. Hydrocortisone acetate was added and uniform suspension was effected using a mixer. The whole volume was made 100 ml by adding purified water. A hydrocortisone acetate suspension for nasal application was thus prepared.

Example 7: Parenteral preparation (injection)

[0059]

Estradiol benzoate	5.0 g
Hydroxypropylcellulose	0.03 g
Chlorobutanol	0.3 g
Sodium chloride	0.9 g
Purified water	q.s. to make 100 ml.

[0060] Chlorobutanol was dissolved in about 80 ml of purified water with warming. Hydroxypropylcellulose was dissolved in the solution by effecting dispersion with warming, followed by cooling to room temperature. Sodium chloride was added for dissolution, estradiol benzoate was added, and uniform suspension was effected using a homogenizer. The whole volume was made 100 ml by adding purified water. An estradiol benzoate suspension for parenteral administration was thus prepared.

Example 8: Preparation for oral administration

[0061]

Mefenamic acid	3.0 g
Methylcellulose	0.01 g
Sorbitol	20 g
5% Ethylparaben solution	1 ml
Purified water	q.s. to make 100 ml.

[0062] Methylcellulose was dissolved in about 50 ml of purified water by effecting dispersion with warming, followed by cooling to room temperature. Sorbitol and 5% ethylparaben solution were added for dissolution. Mefenamic acid was added and uniform suspension was effected using a homogenizer. The whole volume was made 100 ml by adding purified water. A mefenamic acid suspension for oral administration was thus prepared.

Example 9: Lotion

[0063]

Indomethacin	7.5 g
Hydroxypropylcellulose	0.04 g
dl-Camphor	0.5 g
Purified water	q.s. to make 100 ml.

[0064] Hydroxypropylcellulose was dissolved in about 50 ml of purified water by effecting dispersion with warming, followed by cooling to room temperature. dl-Camphor was added for dissolution. Indomethacin was added and uniform suspension was effected ultrasonically. The whole volume was made 100 ml by adding purified water. An indomethacin suspension lotion was thus prepared.

Industrial Applicability

[0065] The aqueous suspension of the present invention has good redispersibility and therefore can be utilized as an excellent aqueous suspension preparation, for example ophthalmic preparation, nasal drops, parenteral preparation, oral preparation, lotion or the like.

Claims

1. An aqueous suspension which comprises a hardly soluble drug together with a water-soluble polymer within a concentration range from the concentration at which the surface tension of the aqueous suspension of said drug begins to decrease up to the concentration at which the reduction in surface tension ceases.

2. The aqueous suspension of Claim 1, wherein the concentration of the water-soluble polymer at which the surface tension of the aqueous drug suspension begins to decrease is 0.00001 to 0.01% (w/v: weight/volume) and the concentration of the water-soluble polymer at which the reduction in surface tension ceases is 0.0001 to 0.1% (w/v).

3. The aqueous suspension of Claim 1, wherein the concentration of the water-soluble polymer is within the range of 0.00001 to 0.1% (w/v).

4. The aqueous suspension of Claim 1, wherein the water-soluble polymer is a cellulose derivative or a water-soluble polyvinyl polymer.

5. The aqueous suspension of Claim 4 wherein the cellulose derivative is at least one member selected from the group consisting of hydroxypropylmethyl cellulose, methylcellulose, hydroxyethylcellulose and hydroxypropylcellulose.

6. The aqueous suspension of Claim 4 wherein the water-soluble polyvinyl polymer is at least one member selected from the group consisting of polyvinylpyrrolidone and polyvinyl alcohol.

7. The aqueous suspension of Claim 3 wherein the ratio of the water-soluble polymer to the hardly soluble drug is 0.0001 to 0.2 part by weight of the former to 1 part by weight of the latter.

8. The aqueous suspension of Claim 1, said suspension being a topical ophthalmic preparation.

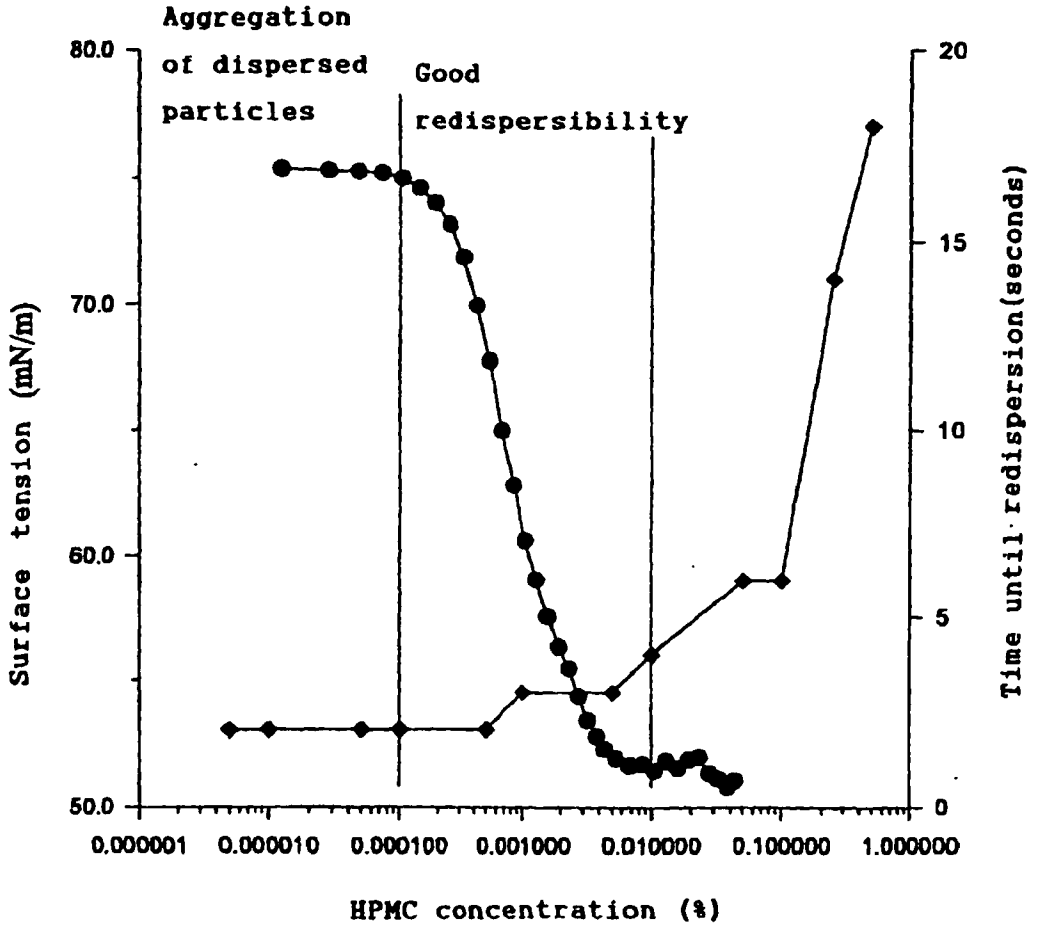
9. The aqueous suspension of Claim 1, said suspension being a topical nasal preparation.

10. The aqueous suspension of Claim 1, said suspension being a parenteral preparation for injection.

11. The aqueous suspension of Claim 1, said suspension being a preparation for oral administration.

12. The aqueous suspension of Claim 1, said suspension being a lotion.

Fig. 1


Aggregation
of dispersed
particles
Good
redispersibility
80.0
70.0
60.0
50.0
20
15
10
5
0
Surface tension (mN/m)
Time until redispersion(seconds)
0.000001
0.000010
0.000100
0.001000
0.010000
0.100000
1.000000
HPMC concentration (%)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP98/01998

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl⁶ A61K9/10, A61K47/32, 47/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl⁶ A61K9/10, 9/107, A61K47/30-47/38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 52-96721, A (Nippon Kayaku Co., Ltd.), August 13, 1977 (13. 08. 77), Claims ; page 2, lower left column, line 16 to page 3, upper left column, line 9 ; page 3 ; Example 2 (Family: none)	1-4, 7
A	JP, 5-186348, A (Takeda Chemical Industries, Ltd.), July 27, 1993 (27. 07. 93) & WO, 9217174, A1 & EP, 531529, A1 & US, 5366985, A	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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(54) Liposome composition and its production.

(57) The liposome compositions entrapping a drug are prepared by constituting the liposomal membrane with saturated phospholipids and aniomic surfactants of high Krafft point at concentrations above their critical micelle concentrations. Thus obtained compositions circulate stably in blood for a long time after intravenous administration.

EP 0 280 492 A2

Description

Liposome Composition and Its Production

This invention relates to liposome compositions and the method for their production.

The idea of Drug Delivery System (DDS) that a drug is targeted to a specified site by intravenous injection of a liposome preparation entrapping the drug has already been generalized [G. Gregoriadis et al., Receptor-mediated targeting of drugs, Plenum Press, New York, p 243-266(1980)]. The characteristic required primarily for such a liposome preparation in DDS is that liposome injected intravenously is stable for prolonged time in blood. However, liposome in itself is not very stable in blood because of interaction with the lipid, a component of the membrane, and the components in blood such as lipoprotein. In addition, liposome injected intravenously is recognized as a foreign substance by the reticulo-endothelial system (RES) on the basis of its physical properties and biochemical characteristics, so that it is apt to be eliminated from blood. Thus, liposome entrapping a drug injected intravenously is eliminated from blood rapidly contrary to expectation. Therefore, it has been an important subject how to stabilize liposome in blood and avoid the recognition by RES so as to prolong the clearance in blood. For example, there is a report that the stability of liposome in blood increases by addition of cholesterol as a componet of the liposomal membrane [C.G. Knight, "Liposomes; from physical stracture to therapeutic applications", Elsevier, North Holland p 310-311(1981)]. However, it may be said that the effect of the addition depends greatly on the original composition of the liposomal membrane [Biochemical et Biophysica Acta, 839, 1-8(1985)]. There is also another report that delivery to RES can be controlled by coating the surface of the liposomal membrane with a glycoprotein containing sial groups as a component of the liposomal membrane [Chem. Pharm. Bull., 34, 2979-2988 (1986)]. There is a report to the contrary that such a glycoprotein containing sialic acid is delivered much to the liver, an organ of RES [Biochemica et Biophysica Acta, 497, 760-765 (1977)].

On the other hand, there is few report that a surfactant was used as a component of the liposomal membrane. The reason is that surfactants are generally considered to unstabilize the structure of the liposomal membrane [Cell Technology (Saibo Kougaku), 2, 1136 (1983)], and rather frequently used to break the membrane [Biochemica et Biophysica Acta, 551 295 (1979)]. Probably the only known method for preparation of liposome using a surfactant is that a homogeneous mixture of an ionic surfactant and a lipid is suspended in an aqueous phase at a concentration below the critical micelle concentration of the surfactant in the aqueous phase, to give a unilamellar liposome [the gazette of Japanese Unexamined Patent Publication No.89633/1984]. Liposome preparations obtained according to this method are, when given intravenously, rapidly eliminated from blood, and the purpose of DDS is not always fulfilled satisfactorily.

As described above, although the idea that liposome entrapping a drug is utilized for DDS by intravenous injection has been known, the liposome preparations produced by the conventional methods are rapidly eliminated from blood after intravenous injection; practically effective means to attain the purpose of DDS have not been developed yet.

Under these circumstances, the inventors investigated various methods to circulate liposome compositions stably for a prolonged time in blood. As a result the inventors found out that the liposome compositions prepared by constituting the liposomal membrane with phospholipids containing saturated acyl groups in the presence of certain surfactants, i.e. anionic surfactants of Krafft point of 37°C or more, are stable in blood, and have completed this invention, after further researches.

Namely this invention relates to (1) liposome compositions produced by entrapping a drug in liposome of which membrane is constituted by a phospholipid having saturated acyl groups and an anionic surfactant of Krafft point of 37°C or more, and

(2) the method for production of liposome compositions entrapping a drug characterized in that membrane of said liposome is constituted by using an emulsion or a suspension prepared with a phospholipid having saturated acyl groups and an aqueous medium of an anionic surfactant of Krafft point of 37°C or more at the concentration above the critical micelle concentration.

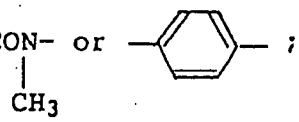
The phospholipids of which acyl groups are saturated acyl groups used for production of the liposome compositions of this invention (sometimes abbreviated simply as phospholipids hereinafter) include glycerophospholipids and sphingophospholipids of which acyl groups are saturated acyl groups. Such phospholipids include those of which two acyl groups are saturated alkyl groups having 8 or more carbon atoms each, at least one of which is a saturated alkyl group having 10 or more, preferably 12-18 carbon atoms. More desirably are used those of which both saturated acyl groups are saturated alkyl groups having 12-18 carbon atoms each. Such phospholipids include hydrogenated lecithine obtained by hydrogenation of lecithin of animal and plant origin (e.g. egg-yolk lecithin , soybean lecithin), and semisynthetic phospholipids obtained by combination of lauroyl, myristoyl, palmitoyl, stearoyl, etc. such as phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerine, phosphatidyl inositol, and sphingomyelin. In more detail, those of which phase transition temperature is usually in the range of about 20-80°C are used preferably. For example those of which observed phase transition temperature is shown in the parentheses described below are used, such as dimyristoylphosphatidyl choline (DMPC 23.9°C), palmitoylmyristoylphosphatidyl choline (PMPC, 27.2°C), myristoylpalmitoylphosphatidyl choline (MPPC, 35.3°C), dipalmitoylphosphatidyl choline (DPPC, 41.4°C), stearoylpalmitoylphospha tidyl choline (SPPC, 44.0°C), palmitoylstearoylphosphatidyl choline (PSPC, 47.4°C), distearoylphosphatidyl choline (DSPC, 54.9°C), dimyristoylphosphatidyl ethanolamine (DMPE, 50°C),

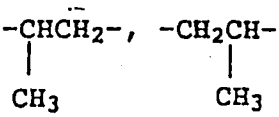
dipalmitoylphosphatidyl ethanolamine (DPPE, 60°C), distearoylphosphatidyl ethanolamine (DSPE, above 60°C), dimyristoylphosphatidyl serine (DMPS, 38°C), dipalmitoylphosphatidyl serine (DPPS, 51°C), distearoylphosphatidyl serine (DSPS, 50°C or more), dimyristoylphosphatidyl glycrine (DMPG, 23°C), dipalmitoylphosphatidyl glycerine (DPPG, 41°C), distearoylphosphatidyl glycerine (DSPG, 55°C), dipalmitoyl sphingomyelin (DPSM, 41°C), and distearoyl sphingomyelin (DSSM, 57°C).

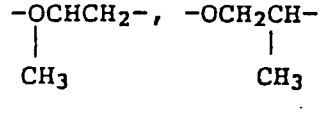
As the anionic surfactants of Krafft point of 37°C or more used in this invention (sometimes abbreviated simply as anionic surfactants hereinafter) those having sulfate group or sulfonate group are advantageously used. The surfactants of Krafft point of 37°C to 90°C are more preferably used in this invention. Such anionic surfactants include those represented by the following general formula:

R-Xm-(Y_1 or Y_2)$_n$-Z

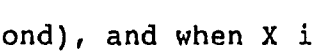
wherein, R is an alkyl group having 12 or more carbon atoms which may be substituted with a sulfate group;

X is -CONH-, -COO-,  or ;

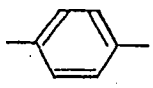
Y_1 is $-CH_2CH_2-$,  or $-CH_2CH_2CH_2-$;

Y_2 is $-OCH_2CH_2-$,  or $-OCH_2CH_2CH_2-$;

Z is $-SO_3^-M^+$, or $-SO_4^-M^+$, (M is an alkali metal element); m is 0 (direct bond) or 1; and n is an integer from 0 (direct bond) to 2, provided that when X is -CONH- or



-CON-, Y_2 is 0 (direct bond), and when X is

 , n is 0 (direct bond).

The alkyl group represented by R usually has 12 to 25 carbon atoms, and those substituted with sulfate group are preferably those having 16 to 25 carbon atoms each, in which the sulfate group may be paired with an alkali metal ion (sodium, potassium, lithium) as the couple ion.

In the following examples of the anionic surfactants are shown.

Anionic surfactants having sulfate group include salts of alkyl sulfate esters such as sodium hexadecyl sulfate (Krafft point; 43°C) and sodium octadecyl sulfate (Krafft point; 58°C); salts of alkyl disulfate esters such as sodium hexadecyl disulfate ester (Krafft point; 39°C) and sodium octadecyl disulfate ester (Krafft point; 45°C); salts of alkylether sulfate esters such as sodium octadecylether sulfate ester (Krafft point; 46°C) and sodium octadecyl diether sulfate ester (Krafft point; 40°C); and salts of fatty acid alkanolamide sulfate esters such as sodium palmitoyl ethanolamide sulfate ester (Krafft point; 42°C), sodium stearoyl ethanolamide sulfate ester (Krafft point; 53°C), sodium palmitoyl propanolamide sulfate ester (Krafft point; 47°C), and sodium stearoyl propanolamide sulfate ester (Krafft point; 57°C). Anionic surfactants having sulfonate group include salts of alkanesulfonic acids such as sodium dodecanesulfonate (Krafft point; 38°C), sodium tetradecanesulfonate (Krafft point; 48°C), sodium pentadecanesulfonate (Krafft point; 48°C), sodium hexadecanesulfonate (Krafft point; 57°C), sodium heptadecanesulfonate (Krafft point; 62°C), and sodium octadecanesulfonate (Krafft point; 70°C); salts of alkylbenzenesulfonates such as sodium dodecylbenzene-

sulfonate (Krafft point; 40°C), sodium tetradecylbenzenesulfonate (Krafft point; 43°C), sodium hexadecylbenzenesulfonate (Krafft point; 46°C), and sodium octadecylbenzenesulfonate (Krafft point; 56°C); salts of acyloxyethanesulfonic acids such as sodium myristoyloxyethanesulfonate (Krafft point; 39°C, sodium palmitoyloxyethanesulfonate (Krafft point; 51°C), and sodium stearoyloxyethanesulfonate (Krafft point; 51°C); and salts of acyltaurines such as sodium palmitoyltaurine (Krafft point; 43°C), sodium stearoyltau rine (Krafft point; 58°C), sodium palmitoylmethyltaurine (Krafft point; 43°C), and sodium stearoylmethyltaurine (Krafft point; 58°C). Among these anionic surfactants, salts of acyltaurines or acylmethyl taurines are particularly preferably used because of their high stability in blood of the liposome preparations prepared and their industrial supply.

In the following, the method for production of the liposome compositions of this invention is described.

The liposome compositions of this invention are prepared so that the phase transition temperature may generally be in the range of about 37 to 60°C, preferably about 40 to 55°C. Adjustment of the phase transition temperature can be achieved by adequate selection of the kind and the combination ratio of phospholipids and anionic surfactants used. The phase transition temperatures of the liposomal membrane thus combined can be confirmed by calorimetry such as DSC (Differential Scanning Calorimeter) or by measurement of the amount released of the drug entrapped in liposome. To adjust the phase transition temperature of the liposomal membrane in the range described above, about 0.5-50 weight parts, preferably about 5-20 weight parts of an anionic surfactant is used per 100 weight parts of a phospholipid. By adjusting the phase transition temperature of the liposomal membrane in the range described above, the purpose of this invention that the clearance in blood of the liposome compositions obtained is prolonged can be attained advantageously.

For composition of the liposome preparations of this invention, aqueous mediums containing anionic surfactants at the concentrations above their critical micelle concentrations are used. The critical micelle concentrations can be determined by usual methods, for example by investigating the relation between physical properties such as surface tension, osmotic pressure coefficient, and electric conductivity, and the concentration of the anionic surfactant [Masayuki Nakagaki and Naofumi Koga, "Drug Physicochemistry (Yakuhin Butsurikagaku)", 3rd Edn., p111 (1969), Nankodo]. Therefore, aqueous mediums can be prepared by using these measurements as indices so that the concentration of the anionic surfactant may be above the critical micelle concentration and the ratio of the amount of the anionic surfactant to that of the phospholipid may be in the range described above. These aqueous mediums may be prepared by dissolving an anionic surfactant in an aqueous medium at a temperature higher than the Krafft point, or by suspending at a temperature lower than the Krafft point. It is desirable that a water-soluble drug is dissolved in these aqueous solutions, and, if necessary, other additives (e.g. sugars and salts as osmotic pressure modifiers, buffers as pH modifiers) may be dissolved. The content of a drug depends on the purpose of the therapy and the potency of the drug.

From the aqueous mediums thus obtained and phospholipids, emulsions or suspensions are prepared to constitute liposome according to the per se known methods for preparations of REV, MLV, SUV and other liposome preparations. For example, liposome compositions from emulsions are prepared as follows; first a phospholipid is dissolved in an organic solvent (e.g. diethylether, isopropylether, chloroform, which are used separately or in combination), to which the aqueous medium of an anionic surfactant described above is added, and a w/o type emulsion is prepared by a conventional method. From this w/o type emulsion, a liposome composition is prepared according to the method described in Proc. Natl. Acad. Sci. USA, 75, 4194 (1978), or the method described in the gazette of Japanese Unexamined Patent Publication No.118415/1980. The amount of an organic solvent used for preparation of emulsion is generally 2-10 times the amount of liquid to be included. The amount of phospholipid is about 10-100 μmol per 1 ml of the liquid to be included, and it is generally desirable that the phospholipid is dissolved in the organic solvent beforehand.

For emulsification to obtain a w/o type emulsion, the conventional methods are applicable such as stirring, pressurization, and sonication. Homogenous emulsions can be obtained by sonication for about 1 to 20 minutes with a 20KHz probe type sonicator. In the method of this invention using an anionic surfactant, emulsification is easy and a homogenous fine emulsion can be obtained.

From these w/o type emulsions thus obtained, the solvent is removed according to the conventional methods. For example, the solvent can be evaporated off with a rotary evaporator. Evaporation is performed desirably at a temperature of 40°C or more, under reduced pressure of about 60-400 mmHg in the initial stage and about 100-700 mmHg after the content has formed a gel. Further evaporation to remove the solvent will give a REV (reverse-phase evaporation vesicle) liposome preparation. This liposome constitutes unilamellar or oligolamellar (usually a lipidic double membrane consisting of about 10 or less layers) entrapping a drug.

On the other hand, liposome preparations of multilamellar vesicles (MLV) type can be obtained by evaporating the organic solvent from the solution of a phospholipid in an organic solvent prepared similarly as described above under reduced pressure to form a thin film of the phospholipid, followed by adding an aqueous medium of an anionic surfactant containing a drug and allowing to disperse at 40°C or more. The MLV preparation thus obtained is shaken with a probe type sonicator to give a liposome preparation of small unilamellar vesicle (SUV) type.

The method for production of liposome compositions of this invention is applicable also to stable plurilamellar vesicle (SPLV) method (the gazette of Japanese Unexamined Patent Publication No.500952/1984) and the dehydration-rehydration vesicle method [C. Kirby et al., Biotechnology, Nov., 979 (1984)]. In the case of a drug that is fat soluble but slightly soluble in water, a liposome composition can be

obtained by entrapping the drug which has been dissolved in the solution of a lipid in an organic solvent as described above. Such a liposome preparation may be used as it is, or, if necessary, after preparations of particles of desirable size by, for example, nuclepore filter or gel filtration. It is desirable to eliminate the free unentrapped drug prior to use by, for example, centrifugation, gel filtration, or dialysis.

The drugs used in this invention are not particularly defined as far as they are used for DDS, including antitumor agents such as platinum compounds (e.g. cisplatin, carboplatin, spiroplatin), adriamycin, mitomycin C, actinomycin, ansamitocin, bleomycin, 5-FU, and methotrexate; lymphokeins such as natural and gene recombinant interferons (α, β, γ), and natural and gene recombinant interleukins; physiologically active peptides such as manganese superoxide desmutase (SOD) and its derivative superoxide desmutase PEG (PEG-500) (the gazette of Japanese Unexamined Patent Publication No.16685/1983 and EPC Patent Application Laid-Open No.0210761); beta lactam antibiotics such as sulfazecin; amino glycoside antibiotics such as gentamycin, streptomycin, and kanamycin; vitamins such as cyanocobalamin and ubiquinone; antiprotozoal agents such as meglumine antimonate; enzymes such as alkali phosphatase; anticoagulants such as heparin; antiallergic agents such as amiexanox; immunoactivating agents such as muramyl dipeptide, muramyl tripeptide, and TMD-66 [Cancer (Gann 74 (2), 192-195 (1983)]; agents for circulatory system such as propranolol; and metabolism activating agents such as glutathione. This invention is preferably applicable particularly to the water soluble drugs because of the purpose of the invention. Such drugs include those of which logarithm of the partition coefficient between octanol and water is 10 or less. The drug is used in an effective amount of objective drugs.

The liposome compositions of this invention are used by intravenous administration by injection or by drip infusion of a suspension of an appropriate amount of a solution or emulsion in for example physiological saline according to the purpose of the therapy.

The liposome compositions of this invention are characterized by the use of saturated phospholipids and anionic surfactants of high Krafft point at concentrations above their critical micelle concentrations.

The liposome compositions of this invention circulate through the body stably in blood for a long time after intravenous administration, which is useful to reduce the toxicity of the drug, enhance the targeting effect of the drug to a specified tissue, and increase the persisting therapeutical effect of the drug. Particularly the liposome compositions of this invention including antitumor agents are expected to enhance the therapeutical effect when administered during hyperthermia; for this purpose, liposome preparations of which phase transition temperature of the liposomal membrane is in the range of about 40-55° C are preferably used.

Brief Description of the Drawings:

Figs. 1, 2 and 3 show the relation between the time after intravenous administration of the liposome composition entrapping 6-CF in rat in Experimental Example 1-2 and the 6-CF blood level. Fig.4 shows the relation between the time after intravenous administration of the liposome composition entrapping 6-CF or CDDP in rat in Experimental Example 2-2 and the blood level. The blood level is shown as % of dose on the assumption that blood volume is 10% of body weight.

Examples

In the following this invention is illustrated in the concrete in Examples, Test examples, and Experimental Eamples.

Example 1

270 mg of DPPC and 30 mg of DSPC were dissolved in 70 ml of 1:1 mixture of chloroform and isopropylether in a 1-1 beaker. To 10 ml of an aqueous solution of 6-carboxyfluorescein (6-CF), pH 7, which had been prepared beforehand so that the osmotic pressure might be the same as that of the physiological saline, 30 mg of sodium stearoylmethyltaurine (SMT) was added at room temperature. SMT remained almost insoluble, but was dissolved rapidly by forming micelles at a temperature above the Krafft point. Then this solution was added to the solution of the phospholipid in an organic solvent, and emulsified by a prove type sonicator (Ohtake, Japan), to five a w/o type emulsion. Sonication at 50 watt for 30 seconds was repeated 10 times. This emulsion was charged to a rotary evaporator to evaporate off the organic solvent at 60° C under reduced pressure, to give REV. The pressure in the evaporator was reduced greatly in the initial stage, but then adjusted in the course of evaporation of the organic solvent to prevent bumping. A small amount of the organic solvent remained in REV was further evaporated off by blowing nitrogen gas. Then an appropriate amount of physiological saline was added to REV to make 10 ml, filtrated through a 1.2 micron filter (Acrodisc, Gelman), and dialyzed through a dialysis membrane (Spectrapor, Spectrum Medical) against physiological saline for 24 hours, to give a liposome compostition entrapping 6-CF. The entrapment ratio of 6-CF in this liposome composition was 33.2% as determined by quantification of 6-CF entrapped in liposome (Note 1).

(Note 1) Quantification of 6-CF in liposome and calculation of entrapment ratio

0.1 ml of a liposome composition is diluted 100 times with phosphate bufferized physiological saline (PBS, pH 7.2), then further diluted 100 times with 0.02 % Triton X-100-containing PBS, heated at 60° C for 30 minutes to break liposome; total 6-CF amount in the liposome suspension was determined by measurement of the intensity of fluorescence of the solution (Hitachi, F3000 Fluorospectrometer, excitation wave length 494 nm, measurement wave length 515 nm). Separately 0.1 ml of the liposome composition is diluted 10000 times with

PBS, 2.5 ml of which was filtrated through a centrifugal filter (Centrisart, SM13249E, Sartorius), and the amount of free unentrapped 6-CF in the liposome suspension was determined by measurement of the intensity of fluorescence of the filtrate.
entrapment ratio =
[(total amount of 6-CF in liposome composition) - (amount of free 6-CF in liposome composition)]/(amount of 6-CF used for preparation of liposome composition) × 100

Example 2
15 mg of SMT was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 34.9%.

Example 3
45 mg of SMT was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 39.4%.

Example 4
60 mg of SMT was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 46.3%.

Example 5
30 mg of sodium palmitoylmethyltaurine (PMT) was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 32.3%.

Example 6
30 mg of sodium octadecanesulfonate (ODS) was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 33.3%.

Example 7
15 mg of ODS was used in place of 30 mg of ODS in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 24.1%.

Example 8
45 mg of ODS was used in place of 30 mg of ODS in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 38.3%.

Example 9
60 mg of ODS was used in place of 30 mg of ODS in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 40.1%.

Example 10
210 mg of DPPC and 90 mg of DSPC were used in place of 270 mg of DPPC and 30 mg of DSPC in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 24.1%.

Example 11
300 mg of DPPC was used in place of 270 mg of DPPC and 30 mg of DSPC in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 35.2%.

Example 12
210 mg of DPPC and 90 mg of DSPC were used in place of 270 mg of DPPC and 30 mg of DSPC in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 28.3%.

Example 13
300 mg of DPPC was used in place of 270 mg of DPPC and 30 mg of DSPC in Example 6 and treated similarly as in Example 1, to give a liposome composition of entrapment ratio of 34.6%.

Example 14
30 mg of sodium palmitoyltaurine (PT) was used in place of 30 mg of SMT in Example 1 and treated similarly as in Example 1, to give a liposome composition of the 6-CF entrapment ratio of 22.4%.

Example 15
360 mg of DPPC and 40 mg of DSPC were dissolved in 40 ml of chloroform in a 1-l beaker. The organic solvent was evaporated off in a rotary evaporator and a lipidic film formed on the glass wall. A trace of the organic solvent remaining in the film was removed by blowing nitrogen gas. To the film thus prepared 10 ml of 6-CF solution containing 40 mg of SMT used in Example 1, which had been kept at 60°C, was added at 60°C, and allowed to disperse by vortex, to give a MLV liposome. The MLV preparation thus obtained was treated by

sonication at 50 watt for about 10 minutes with the probe type sonicator used in Example 1, to give a SUV. Similarly as in Example 1, the preparation was further subjected to filtration and dialysis, to give a liposome composition of 6-CF entrapment ratio of 5.7%.

Example 16

280 mg of DPPC and 120 mg of DSPC were used in place of 360 mg of DPPC and 40 mg of DSPC in Example 15 and treated similarly as in Example 15, to give a liposome composition of the 6-CF entrapment ratio of 6.3%.

Example 17

400 mg of DPPC was used in place of 360 mg of DPPC and 40 mg of DSPC in Example 15 and treated similarly as in Example 15, to give a liposome composition of the 6-CF entrapment ratio of 6.0%.

Example 18

40 mg of PMT was used in place of 40 mg of SMT in Example 15 and treated similarly as in Example 15, to give a liposome composition of the 6-CF entrapment ratio of 6.8%.

Example 19

40 mg of ODS was used in place of 40 mg of SMT in Example 15 and treated similarly as in Example 15, to give a liposome composition of the 6-CF entrapment ratio of 6.5%.

Example 20

40 mg of sodium palmitoyltaurine (PT) was used in place of 40 mg of SMT in Example 15 and treated similarly as in Example 15, to give a liposome composition of the 6-CF entrapment ratio of 6.0%.

Experimental Example 1-1

Control liposome compositions were prepared which did not contain anionic surfactants and corresponded respectively to the liposome compositions obtained in Examples 1, 10, 11, 15, 16 and 17 described above. Another control liposome composition was prepared in a similar manner as in Example 1 by using 200 mg of egg-yolk phosphatidyl choline containing unsaturated acyl groups, 100 mg of cholesterol, and 30 mg of SMT in place of 270 mg of DPPC, 30 mg of DSPC, and 30 mg of SMT used in Example 1. A SMT-Free control liposome composition corresponding to this preparation was prepared. Another control liposome composition was prepared by using sodium dodecyl sulfate (SDS, Krafft point; 9° C) in place of SMT used in Example 15.

Experimental Example 1-2

The liposome composition obtained in Example 1 described above, and the liposome composition prepared similarly but without the anionic surfactant, 0.1-0.5 ml each, were given intravenously in rat, and elimination from blood was investigated (Note 2); the results are shown in Fig.1. As shown in Fig.1, the blood levels of the liposome containing the anionic surfactant (-●-) were much higher than those of the control liposome without the surfactant (....X....). The liposome compositions obtained in Examples 1, 6, 10, 15, 18, 19 and 20, 0.1-0.5 ml each, were given intravenously in rat, and the amount of liposome remaining in blood one hour after administration was 9.7, 11.9, 26.4, 2.7, 2.3, 2.8, and 2.2 times as much as that of the respective control liposome prepared similarly but without the anionic surfactant. On the other hand, as shown in Fig.2, the anionic surfactant-containing liposome prepared from egg-yolk phosphatidyl choline and cholesterol (-●-) was eliminated from blood as rapidly as the control liposome (....X....). Fig. 3 shows the elimination from blood after intravenous administration in rat of 0.2 ml either of the SDS-containing liposome obtained in Experimental Example 1-1 described above (....X....) or of the liposome prepared similarly but without SDS (-●-). The results shown in these Figs. clearly indicate that the liposome compositions of this invention prepared by using a phospholipid containing saturated acyl groups and an anionic surfactant of Krafft point of 37° C or more as the components of the liposomal membrane are characterized by the much prolonged elimination time from blood after intravenous administration as compared with the control liposome compositions.

Experimental 1-3

The liposome compositions obtained in Examples 1, 15, 18, and 19, and Experimental Example 1-1 described above were given intravenously in rat, and the level of 6-CF in liver was determined to know the delivery of liposome to RES (Note 2); the results are shown in Table 1. These results indicate that the elimination time of liposome from blood was prolonged and delivery to RES such as the liver was reduced.

Table 1 Liposome level in liver one hour after administration (%)

liposome preparation	with anionic surfactant	without anionic surfactant
Example 1	16.7	30.1
Example 15	16.9	44.7
Example 18	19.1	44.7
Example 19	15.0	44.7

(Note 2) Measurement of 6-CF liposome level in blood and in liver

To 0.2 ml of heparinized blood drawn from the caudal vein, 10 ml of PBS was added to give a blood suspension. This suspension was centrifuged (3000 rpm, 10 minutes), and to 5 ml of the resultant supernatant 0.05 ml of Triton X-100 was added and heated at 60-70°C to break liposome; the liposome level in blood was determined by measurement of fluorescence of 6-CF released. The liver extirpated after abdominal section and exsanguination was immersed in PBS containing 0.02 % Triton X-100 to make 100 ml. The tissue was homogenized by a homogenizer (Polytron, Kinematica), and heated at 60-70°C, to give a homogenate in which all of the 6-CF had been released. The homogenate was subjected to ultracentrifugation (50000g, 10 minutes), diluted 20-50 times, and filtrated through a 0.45 micron membrane filter (Acrodisk, Gelman); the liposome level in liver was determined by measurement of the fluorescence of the filtrate.

Experimental Example 1-4

Heat release from 10000 times dilutions with PBS (2% plasma) of the liposome compositions obtained in Examples 1, 2, and 6, and the respective control prepara tions containing no anionic surfactants was investigated by continuous measurement of the amount of 6-CF released from liposome with a fluorometer connected to a programmed temperature system, to follow the phase transition of liposomal membrane (change from gel to liquid crystal). The initiation temeperature of heat release and the phase transition temperature determined from the heat release curve are shown in Table 2. The phase transition temperature was measured with a thermal analyzing system (SEIKO I & E, SSC 50000 type, 2°c/min). Both temperatures are closely related to each other.

Table 2 Phase transition temperature (°C) of liposomal membrane and heat release initiation temperature (°C) of 6-CF from liposome

liposome composition	phase transition temperature	heat release initiation temperature
Example 1	42.3	36.1
Example 6	43.7	39.3
Example 10	42.8	36.3
without surfactant	41.9	37.9

Example 21

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution obtained in Example 1 and treated similarly as in Example 1, to give a liposome composition of the CDDP entrapment ratio of 23.5% and the phase transition temperature of 42.7°C.

(Note 3) Measurement of CDDP content in liposome

0.1 ml of a liposome composition was suspended in 5 ml of physiological saline, and 2.5 ml of the suspension was freeze-dried and heated; the resultant solution containing broken liposome, about 2.5 ml, was filtrated

through Centrisalt, and to 0.1 ml of the filtrate 2 ml of 0.1 N NaOH solution containing 10% of diethyldithiocarbamate (DDTC) was added and kept at room temperature for 30 minutes; the resultant adduct was extracted with 5 ml of n-hexane and the extract was analyzed by HPLC (column; Zorbax CN, solution; n-hexane/isopropylalcohol = 8/2; UV=250 nm) to determine the total CDDP amount in the liposome suspension. Separately the remaining solution of liposome in physiological saline, about 2.5 ml, was filtrated through Centrisalt and the amount of free CDDP remaining unentrapped in liposome was determined under the same conditions as described above.

Example 22

A 500 μg/ml solution of cisplation (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 2 and treated similarly as in Example 2, to give a liposome composition of the CDDP entrapment ratio of 21.4%.

Example 23

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 3 and treated similarly as in Example 3, to give a liposome composition of the CDDP entrapment ratio of 25.8%.

Example 24

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 5 and treated similarly as in Example 5, to give a liposome composition of the CDDP entrapment ratio of 24.0%.

Example 25

A 500 μg/ml solution of cisplation CDDP) in physiological saline was used in place of the 6-CF solution used in Example 6 and treated similarly as in Example 6, to give a liposome composition of the CDDP entrapment ratio of 21.8% and the phase transition temperature of 43.9°C.

Example 26

A 500 μg/ml solution of cisplatin (CDDP) in physio logical saline was used in place of the 6-CF solution used in Example 7 and treated similarly as in Example 7, to give a liposome composition of the CDDP entrapment ratio of 21.9%.

Example 27

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 8 and treated similarly as in Example 8, to give a liposome composition of the CDDP entrapment ratio of 24.9%.

Example 28

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 10 and treated similarly as in Example 10, to give a liposome composition of the CDDP entrapment ratio of 23.3%.

Example 29

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of the CDDP entrapment ratio of 27.7% and the pahse transition temperature of 41.9°C.

Example 30

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution obtained in Example 12 and treated similarly as in Example 12, to give a liposome composition of the CDDP entrapment ratio of 24.0%.

Example 31

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 13, to give a liposome composition of the CDDP entrapment ratio of 24.5% and the phase transition temperature of 42.5°C.

Example 32

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 14 and treated similarly as in Example 14, to give a liposome composition of the CDDP entrapment ratio of 25.0%.

Example 33

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 15 and treated similarly as in Example 15, to give a liposome composition of the CDDP entrapment ratio of 4.8%.

Example 34

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution obtained in Example 16 and treated similarly as in Example 16, to give a liposome composition of the CDDP entrapment ratio of 5.0%.

Example 35

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 17 and treated similarly as in Example 17, to give a liposome composition of the CDDP entrapment ratio of 5.2%.

Example 36

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 18 and treated similarly as in Example 18, to give a liposome composition of the CDDP entrapment ratio of 4.3%.

Example 37

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 19 and treated similarly as in Example 19, to give a liposome composition of the CDDP entrapment ratio of 4.9%.

Example 38

A 500 μg/ml solution of cisplatin (CDDP) in physiological saline was used in place of the 6-CF solution used in Example 20 and treated similarly as in Example 20, to give a liposome composition of the CDDP entrapment ratio of 4.7%.

Experimental Example 2-1

Liposome compositions containing no anionic surfactants were prepared as the respective control compositions of the liposome preparations obtained in Examples 21, 28, 29, 33, 34, and 35.

Experimental Example 2-2

The 6-CF level in blood until 6 hours after intravenous administration in rat of the liposome composition of Example 1 was compared with the CDDP level (Note 4) after intravenous administration of the liposome composition of Example 15 in rat, and the result is shown in Fig.4. At any time point, the level of CDDP was similar to that of 6-CF, suggesting that the CDDP liposome composition (-●-) may behave similarly to the 6-CF liposome composition (....x....). Also the liposome compositions obtained in Examples 21, 22, 25, 26, and 29 showed values as high as those obtained with the 6-CF liposome compositions. These results also inidicate that the liposome compositions of this invention prepared by using a phospholipid containing saturated acyl groups and an anionic surfactant of Krafft point of 37°C or more are characterized by the much prolonged elimination time from blood after intravenous administration, as compared with the control liposome compositions.

(Note 4) Measurement of CDDP level in blood

To 0.2 ml of heparinized blood obtained from caudal vein, 2 ml of PBS was added to give a blood suspension. To 1 ml of the supernatant obtained by centrifugation of the suspension, 1 ml of DDTC solution was added; the total amount of CDDP in blood was determined similarly to the method of measurement of CDDP amount described above.

Experimental Example 2-3

The SMT content of the composition in Example 21 described above was determined (Note 5), and the result showed that about 90% of the amount charged for preparation of the liposome composition remained. This value was much higher than the CDDP entrapment ratio of 23.5%, indicating that SMT certainly constitutes the liposomal membrane, and that about 150 molecules of SMT are present per 1000 molecules of the phospholipid in the liposomal membrane.

(Note 5) Measurement of SMT content

15 mg of methylene blue, 6 g of concentrated sulfuric acid, and 25 g of anhydrous sodium sulfate were dissolved in distilled water, to make 500 ml of a reaction test solution. To 5 ml of this reaction test solution, 10 ml of a diluted liposome composition (10000 times) and 5 ml of chloroform were added, and allowed to separate into two layers after thorough shaking; absorbance (653 nm) of the chloroform layer was measured. Absorbance of SMT solutions of various concentrations (less than 10 ppm) was measured to make a

calibration curve. A blank test was conducted by using the SMT-free liposome composition in Experimental Example 2-1.

Example 39

A 308 μg protein/ml aqueous solution of interleukin 2 (IL-2) (in 25 mM ammonium acetate solution pH 6) was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention.

Example 40

A 308 μg protein/ml aqueous solution of IL-2 was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 13, to give a liposome composition of this invention.

Example 41

A 100 μg/ml aqueous solution of ansamitocin was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping ansamitocin.

Example 42

A 100 μg/ml aqueous solution of ansamitocin was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 13, to give a liposome composition of this invention entrapping ansamitocin.

Example 43

A 5 mg/ml solution of methotrexate in physiological saline was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping methotrexatae.

Example 44

A 5 mg/ml solution of methotrexate in physiological saline was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 13, to give a liposome composition of this invention entrapping methotrexate.

Example 45

A 200 μg/ml solution of mitomycin C in physiological saline was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping mitomycin C.

Example 46

A 200 μg/ml solution of mitomycin C in physiological saline was used in place of the 6-CF solution used in Example 13, and treated similarly as in Example 13, to give a liposome composition of this invention entrapping mitomycin C.

Example 47

A 1 mg/ml solution of adriamycin in physiological saline was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping adriamycin.

Example 48

A 1 mg/ml solution of adriamycin in physiological saline was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping adriamycin.

Example 49

A 3 mg/ml solution of bleomycin in physiological saline was used in place of the 6-CF solution used in Example 11 and treated similarly as in Example 11, to give a liposome composition of this invention entrapping bleomycin.

Example 50

A 3 mg/ml solution of bleomycin in physiological saline was used in place of the 6-CF solution used in Example 13 and treated similarly as in Example 13, to give a liposome composition of this invention entrapping bellmycin.

Claims

1. Liposome compositions entrapping a drug in liposome of which membrane is constituted by a

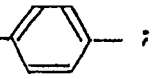
phospholipid of which acyl groups are saturated acyl groups and an anionic surfactant of Krafft point of 37°C or more.

2. The composition according to claim 1, wherein phase transition temperature of the liposomal membrane is in the range of about 37 to 60°C.

3. The composition according to claim 1, wherein the anionic surfactants are those represented by the following general formula:

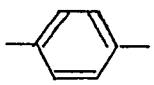
R-Xm-(Y_1 or Y_2)$_n$-Z

wherein, R is an alkyl group having 12 or more carbon atoms which may be substituted with a sulfate group;

X is -CONH-, -COO-, -CON(CH$_3$)- or  ;

Y_1 is $-CH_2CH_2-$, $-CH(CH_3)CH_2-$, $-CH_2CH(CH_3)-$ or $-CH_2CH_2CH_2-$;

Y_2 is $-OCH_2CH_2-$, $-OCH(CH_3)CH_2-$, $-OCH_2CH(CH_3)-$ or $-OCH_2CH_2CH_2-$;

Z is $-SO_3^-M^+$, or $-SO_4^-M^+$, (M is an alkali metal element); m is 0 (direct bond) or 1; and n is an integer from 0 (direct bond) to 2, provided that when X is -CONH- or -CON(CH$_3$)-, Y_2 is 0 (direct bond), and when X is  , n is 0 (direct bond).

4. The composition according to claim 1, wherein the anionic surfactants are salts of acyltaurines or acylmethyltaurines.

5. The composition according to claim 1, wherein the drug is of which logarithm of the partion coefficient between octanol and water is 10 or less.

6. The composition according to claim 1, wherein the drugs are antitumor agents, lymphokines, physiologically active peptides, antibiotics, vitamins, antiprotozoal agents, enzymes, anticoagulants, antiallergic agents, immunoactivating agents, agents for circulatory system or metabolism activating agents.

7. The composition according to claim 6, wherein antitumor agents are platinum compounds.

8. The composition according to claim 7, wherein the platinum compound is cisplatin.

9. The composition according to claim 6, wherein an antitumor agent is entrapped in the liposome of which membrane has a phase transition temperature of about 40 to 55°C.

10. A method of producing lip some composition entrapping a drug, which comprises (1) preparing an aqueous medium an anionic surfactant having Krafft point of 37°C or more at the concentration above the critical micelle concentration, (2) mixing the resulting aqueous medium with a phospholipid of which acyl groups are saturated acyl groups to prepare a emulsion or a suspension, wherein an effective amount of a drug is added in the process (1) and/or (2), and (3) subjecting the resulting emulsion or suspension to preparation of liposome vesicles so that the liposomal membrane is constituted by said surfactant and phospholipid.

11. The method according to claim 10, wherein liposome vesicles are prepared by a method of producing REV, MLV, SUV or SPLV.

12. The method according to claim 10, wherein an amount of the anionic surfactant is in the range of about 0.5 to 50 weight parts per 100 weight parts of the phospholipid.

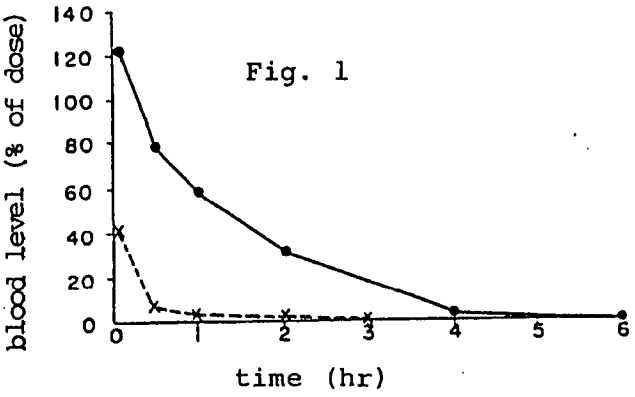


Fig. 1

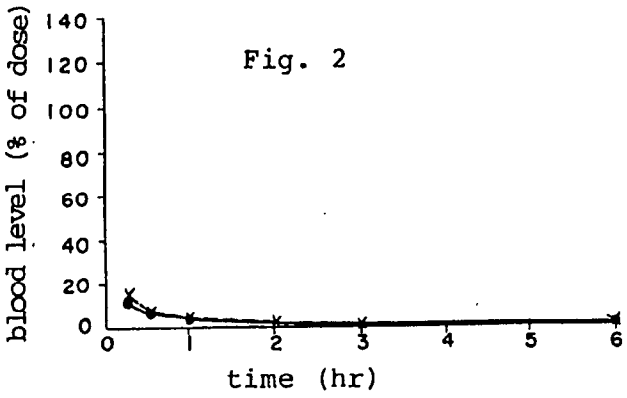


Fig. 2

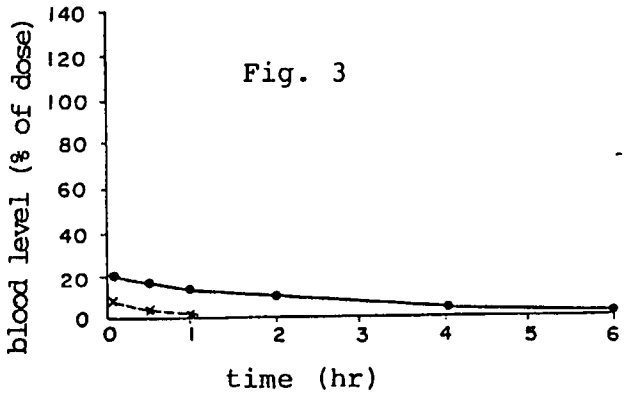


Fig. 3

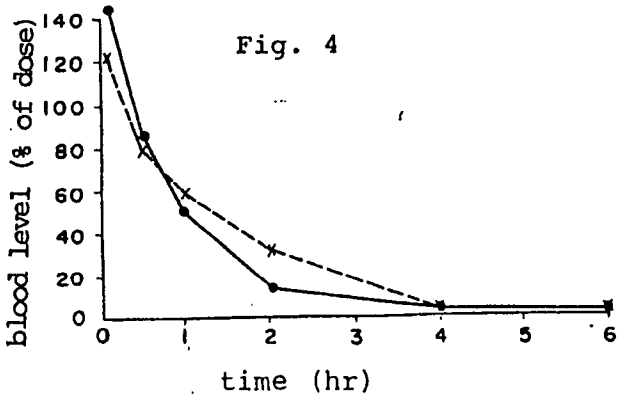


Fig. 4

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Description

FIELD OF THE INVENTION

This invention discloses a pharmacological agent-lipid solution preparation comprising a lipophilic pharmacological agent, a desalted charged lipid, and an aqueous-miscible lipid solvent such that upon introduction into an aqueous medium a suspension of lipid associated with the pharmacological agent is formed. Further disclosed are preparation and methods of manufacture and use of the pharmacological agent-lipid solution preparation, and the co-solubilizing of lipid associating pharmocological agent in lipid and ethanol solution.

BACKGROUND OF THE INVENTION

Lipids are known to be useful as carriers for the delivery of drugs to mammals including humans. In pharmaceutical preparations lipids are variously used as admixtures with drugs or in the form of liposomes.

Liposomes are vesicles comprising closed bilayer membranes containing an entrapped aqueous phase. Liposomes may be any variety of unilamellar vesicles (possessing a single membrane bilayer) or multilamellar vesicles (e.g. onion-like structures characterized by concentric membrane bilayers, each separated from the next by an aqueous layer).

Liposomes are formed by methods well known in the art. The original liposome preparation of Bangham et al. (1965, J. Mol. Biol. 13:238-252) involves suspending phospholipids in an organic solvent which is then evaporated to dryness leaving a phospholipid film on the reaction vessel. Then an appropriate amount of aqueous phase is added, the mixture is allowed to "swell", and the resulting liposomes which consist of multilamellar vesicles are dispersed by mechanical means. The structure of the resulting membrane bilayer is such that the hydrophobic (nonpolar) "tails" of the lipid orient toward the center of the bilayer while the hydrophilic (polar) "heads" orient toward the aqueous phase. This technique provides the basis for the development of the small sonicated unilamellar vesicles described by Papahadjapoulos and Miller (1967, Biochim. Biophys. Acta. 135:624-638) and large unilamellar vesicles.

Another class of liposomes is characterized as having substantially equal interlamellar solute distribution. This class of liposomes is denominated as stable plurilamellar vesicles (SPLV) as defined in U.S. Patent No. 4,522,803 to Lenk et al. and includes monophasic vesicles as described in U.S. Patent No. 4,588,578 to Fountain et al. and frozen and thawed multilamellar vesicles (FATMLV) as described in "Solute Distributions and Trapping Efficiencies Observed in Freeze-Thawed Multilamellar Vesicles," Mayer et al., Biochima et Biophysica Acta. 817: 193-196 (1985).

Another method of liposome formation is by the infusion of lipid solvent such as diethyl ether or ethanol which contains phospholipids into an aqueous solution containing a pharmacological agent resulting in the formation of liposomes which entrap a portion of the aqueous solution. This procedure cannot be used to entrap lipid soluble pharmacological agents soluble in fat or fat solvents due to the very limited solubility of such agents in an aqueous solution.

Lipid soluble pharmacological agents include anti-neoplastics such as doxorubicin; antifungals such as miconazole, terconazole and amphotericin B; immunomodulators such as cyclosporin A; derivatives of muramyl dipeptides such as muramyl tripeptide phosphatidylethanolamine; and, hormones such as glucocorticoids, mineralocorticoids and estrogens; anti-inflammatories such as the steroidals, prednisone, dexamethasone and fluromethasone and the nonsteroidals indomethacin, salicylic acid acetate (aspirin) and ibuprofen, further including analgesic agents such as acemetacin and flurobiprofen; and other agents such as lipoxygenase inhibitors, prostaglandins, neuroleptics, antidepressants, fat-soluble vitamins, contrast materials and antivirals. Pharmacological agents as used herein includes agents administered to animals including mammals, particularly humans, in the course of treatment or diagnosis. Biologically active materials such as drugs as well as diagnostic agents and contrast materials which are usually nonreactive are all to be understood to be pharmacological agents.

Solubilization of lipid soluble pharmacological agent-lipid suspension preparation in water is usually done with the help of solubilizing agents such as polyethylene glycols and propylene glycol, or via surfactants including such well known surfactants as polysorbates, poloxamers, and polyethoxylated castor oil. Upon administration, however, these agents may be present in concentrations sufficient to induce undesirable side effects.

To avoid the use of such agents, D. Schmidt (U.S. Pat. No. 4,271,196) proposed colloidal suspensions formed by solubilization of lipids in ethanol, removal of the solvent by evaporation and addition of water or buffer with the drug added before water or in the colloidal suspension of lipids. Similarly, J. Schrank and H.

Steffen (U.S. Pat. No. 4,411,894) solubilized both lipids and drug in ethanol, then ethanol was removed and buffer was added to form liposomes.

These and other procedures involving the removal of ethanol and liposome formation have two major disadvantages. First, ethanol cannot solubilize certain lipids; in particular, salt forms of acidic, or basic phosphatides ("charged phosphatides") such as phosphatidic acid, dicetylphosphate, phosphatidylethanolamine, and phosphatidylserine. Second, the entrapment of lipophilic drug in liposomes is limited such that the drug/lipid ratio (wt/wt) is usually less than 0.2.

It is to be understood that neutral lipids are those which do not present a charge at neutral pH. Phospatidylcholine having a zwitterionic group is termed a neutral polar lipid and compounds such as cholesterol or triglycerides are nonionizable at physiological pH's and are termed neutral nonpolar lipids.

To increase the efficacy of drug solubilization by the lipids, F. Tsunekazu et al. (European Pat. No. 0161445Al) proposed the solubilization of lipids and drug in an organic solvent, removal of the organic solvent, homogenization of the resulting film in aqueous solution by ultrasonic treatment, centrifugation of the suspension and recovery of the lower most layer of the sediment, to yield a particular drug-phospholipid complex. In this publication, particular reference is made to drugs having a molecular weight below 1,000.

Lipid preparations such as liposomes carrying pharmacological agent-lipid solution agents are often characterized by having insufficient shelf life. Dried liposome preparations have been offered to overcome this problem however such preparations must be reconstituted at the time of use. Reconstitution may be associated with problems of clumping and uncertainty as to the liposomal size of the reconstituted preparation, and uncertainty as to the strength of an aliquot. These preparations are also associated with rapid sedimentation.

It is an object of this invention to provide a pharmacological agent-lipid solution preparation in high drug to lipid ratio.

It is a further object of this invention to provide a pharmacological agent-lipid solution preparation wherein the pharmacological agent is of a molecular weight of greater than 1000.

It is another object of this invention to provide a pharmacological agent-lipid solution preparation sterilizable by filtration.

It is an additional object of this invention to provide a pharmacological agent-lipid solution preparation of lipophilic pharmacological agent.

It is another object of this invention to provide a pharmacological agent-lipid solution preparation that will form a suspension of lipid associated with said pharmacological agent upon introduction into an aqueous medium and further that such suspension exhibit a stability of at least 0.25 to 6 hours or longer without sedimentation and preferably at least 2 hours.

It is a further object of this invention to provide a method of forming such suspension.

SUMMARY OF THE INVENTION

This invention comprises a pharmacological agent-lipid solution preparation comprising a lipophilic pharmacological agent, a desalted charged lipid and an aqueous miscible lipid solvent. This preparation, upon introduction into an aqueous medium, forms a suspension of lipid associated with the pharmacological agent. In some embodiments the pharmacological agent-lipid solution preparation may be in an oral dosage form such as a unit oral dosage form including tablets, capsules, dragees, and troches which is to include methods of treating subjects employing such dosage forms. The suspended lipid associated with pharmacological agent will be termed an aggregate.

This invention further comprises such preparation wherein the desalted charged lipids are desalted charged phosphatides such as phosphatidic acid, dicetylphosphate, phosphatidylethanolamine, and phosphatidylserine.

Further encompassed are pharmacological agent-lipid solution preparations which are both pharmaceutically acceptable and of limited flammability preferably by use of less volatile lipid solvents or by admixture of a first lipid solvent with secondary less flammable solvents such as polyethylene glycol (400-800 mw being preferred) and propylene glycol. Particularly preferred are preparations of at least 10% (wt/wt) polyethylene glycol (800 mw) with 30% most preferred.

Additionally encompassed by this invention is the nonaqueous water-miscible lipid solvent being absolute ethanol.

Further entailed in this invention is the lipid soluble pharmacological agent being an immunomodulator such as cyclosporin A; an anti-neoplastic such as doxorubicin; an antifungal such as miconazole, terconazole, and amphotericin B; an anti-inflammatory such as the steroidal anti-inflammatories prednisone, doxamethasone, fluoromethasone and the nonsteroidal anti-inflammatories such as indomethacin, salicylic

acid acetate, ibuprofen; and the derivatives of muramyl dipeptide such as muramyl tripeptide phosphatidylethanolamine, and hormones such as glucocorticoids, mineralocorticoids and estrogens.

Particularly included in this invention are anti-inflammatories in unit oral dosage form including tablet, capsule, dragee, or troche, and methods of treating subjects employing such dosage forms.

Included in this invention is a preparation wherein the pharmacological agent is indomethacin is present from 0.5% to 30% by weight, and more particularly present at from 10 to 20% by weight, perticularly in unit dosage forms, and also wherein the lipid is additionally comprised of at least 70% by weight phosphatidylcholine.

In another embodiment polypeptides having a molecular weight of greater than 1000, such as cyclosporin A or insulin, are the pharmacological agents.

A further embodiment of the invention is the method of preparing a suspension from the pharmaceutical agent-lipid liquid solution preparation by adding the preparation to pharmaceutically acceptable aqueous medium. This is preferably added at a ratio of at least 0.1:1 v/v. This method utilizes the pharmaceutical agent-lipid liquid preparations with all of the above noted lipids, solvents, and pharmaceutical agents.

The aqueous media used in the method of preparation include water, 5% dextrose in water (wt/v), 0.9% saline, physiological phosphate buffer, and physiological citrate buffer.

Yet further this invention encompasses suspensions of the pharmacological agent-lipid solution preparation in aqueous media wherein the pharmacological agent to lipid ratio is at least about 20 moles percent.

This invention further includes a compostion of treating animals (including humans) in need of such treatment comprising a therapeutically effective amount of pharmacological agent-lipid solution preparation added to a pharmaceutically acceptable aqueous medium thus forming a suspension. This administration is preferably parenteral, intramuscularly, intraperitioneally, intravenously, subcutaneously, or topically, via inhalation, or oral administration including suppository, or ingestion. The pharmacological agents of this method of administration will be any of those noted above and others. The lipids of this method of administration will be any of those noted above and others.

Also included is pharmacological preparation comprising absolute ethanol, lipid, and nonsteroidal anti-inflammatory, particularly wherein the nonsteroidal anti-inflammatory is indomethacin, and wherein the indomethacin is present from 0.5% to 30% by weight and wherein the indomethacin is present at from 10 to 20% by weight.

DETAILED DESCRIPTION OF THE INVENTION

The "pharmacological agent-lipid solution preparations" of this invention first comprise at least one lipophilic pharmacological agent, as well as a lipid solvent, and at least one lipid. Lipophilic (or lipid soluble) as defined herein includes along with true lipid solubility, the capacity to closely associate with lipids.

Lipid soluble pharmacological agents include respiratory agents such as theophyllin, anti-epileptics such as diphenylhydantoin, anti-neoplastics such as doxorubicin; antifungals such as miconazole, terconazole and amphotericin B, (some antifungals will require desalting and or a acidification of the lipid solvent to increase solubility); immunomodulators such as cyclosporin A; derivatives of muramyl dipeptides such as muramyl tripeptide phosphatidylethanolamine; and, hormones such as glucocorticoids, mineralocorticoids and estrogens; anti-inflammatories such as the steroidals, prednisone, dexamethasone and fluromethasone and the nonsteroidals such as indomethacin, salicylic acid acetate and ibuprofen, further including analgesic agents such as acemetacin and flurobiprofen, and other agents such as lipoxygenase inhibitors, prostaglandins, neuroleptics, antidepressants, fat-soluble vitamins, contrast materials and antivirals.

Other preferred nonsteroidal anti-inflammatories are:

carboxylic acids

salicylates

Acetylsalicylic Acid (i.e., Salicyilic Acid Acetate)

Salsalate

Diflunisal

Fendosal

Acetic Acids

Indomethacin

Acemetacin

Cinmetacin

Sulindac

Tolmetin

Zomepirac

Diclofenac
Fenclofenac
Isoxepac
Furofenac
Fentiazac
Clidanac
Oxepinac
Fenclorac
Lonazolac
Metiazinic Acid
Clopirac
Amfenac
Benzofenac
Clometacine
Etodolac
Bumidazone
Clamidoxic Acid

Propionic Acids

Ibuprofen
Flurobiprofen
Naproxen
Ketoprofen
Fenoprofen
Benoxaprofen
Indoprofen
Pirprofen
Carprofen
Oxaprozin
Pranoprofen
Suprofen
Miroprofen
Tioxaprofen
Alminoprofen
Cicloprofen
Tiaprofenic Acid
Furaprofen
Butibufen
Fenbufen
Furobufen
Bucloxic Acid
Protizinic Acid

Fenamates

Mefanamic Acid
Flufenamic Acid
Meclofenamate
Niflumic Acid
Tolfenamic Acid
Flunixin
Clonixin

Pyrazoles

Phenylbutazone and Analogs
Peprazone (Prenazone)
Apazone (Azapropazone)
Trimethazone
Mofebutazone
Kebuzone
Susibuzone

Oxicams

Piroxicam
Isoxicam
Tenoxicam

Indomethacin is a most preferred nonsteroidal anti-inflammatory. In the preparations of this invention, indomethacin preferably comprises from 0.5% to 30% of the final weight of the pharmacological agent-lipid solution preparation, and particularly from 10 to 20%, and most particularly 15%.

It is a limitation of this invention that at least one lipid be charged and desalted, and such desalted charged lipids are (a) soluble in the water miscible lipid solvents of this invention and (b) exhibit only a limited tendency for sedimentation upon dispersal in the aqueous medium of suspension formation.

Lipid materials used in this invention are amphipathic in character. Amphipathic as defined herein is a moiety with a hydrophobic portion and a hydrophilic portion. Hydrophilic character will be imparted to a molecule through the presence of phosphatidic, carboxylic, sulphatic, amino, sulfhydryl, nitro, and other groups such as carbohydrates. Hydrophobicity will be conferred by the inclusion of groups that include, but are not limited to, long straight and branched chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group. The preferred amphipathic compounds are phosphoglycerides, representative examples of which include phosphatidylcholines, phosphatidylethanolamines, lysophosphatidylcholines, lysophosphatidylethanoloamines, phosphatidylserines, phosphytidylinositols, phosphatidic acids, phosphatidylglycerols and diphosphatidylglycerols as well as sphingomyelins. Synthetic saturated compounds such as dimyristeoylphosphatidylcholine and dimyristoylphosphatidylglycerol or unsaturated species such as dioleoylphosphatidylcholine or dilinoleoylphosphatidylcholine are also usable. Other compounds lacking phosphorous, such as members of the glycolipids, and glycosphingolipid, ganglioside and cerebroside families, are also within the group designated as amphipathic lipids. Salts of acid derivatives of sterols and tocopherols such as cholesterol or tocopherol hemisuccinate are also amphipathic. Ionic detergents such as octadecanylsulfonate are also included.

Salts of acidic or basic lipids (i.e., charged lipids) that otherwise are not soluble in ethanol can be rendered soluble by desalting, that is by removal of the counterion. For example phosphatidic acid, phosphatidylserine, dicetylphosphate, phosphatidylglycerol and phosphatidylethanolamine may be desalted. Natural soy or egg phosphatides may be desalted and the resulting desalted mixture of various lipids will contain sufficient desalted charged lipids in the form of acidic phospholipids for use in this invention. Thus in the practice of this invention, the presence of an amount of neutral lipids, polar or nonpolar, along with desalted charged lipids will not adversely affect the preparation. The tolerable amount of neutral lipid is limited by the solubility of the various lipids in water-miscible lipid solvent system and the required stability of the suspension formed upon mixture with the aqueous medium. Thus, a critical element of this invention is the presence of a desalted charged lipid. The minimum amount of desalted charged lipid will vary with the system. However, at minimum at least sufficient desalted charged lipid must be present to form a stable suspension. Each system will present a different minimum amount of charged lipid necessary for stability but, by way of example, desalted phosphatidic acid will be effective in a presence as low as 0.5 mole percent relative to total lipid in a cyclosporin A-ethanol system. The desirable amount of desalted charged lipid for other systems is easily determined with reference to the solubility of proposed lipid in the solvent system and the required stability of the final aqueous suspension.

Desalting of lipids is accomplished by exchanging the counterion from the acidic or basic moiety of the amphipathic lipid for a proton or hydroxide, respectively. This is done by any of a number of methods well known in the art including ion exchange resin column elution.

The typical ion exchange resin procedure employs commercially available resins such as those of the Biorad Company of Richmond, Virginia. A typical cation exchange resin is Biorad AG50-X8® and Biorad AG1-X8® is a typical anion exchange resin. These procedures, performed in the aqueous-soluble lipid solvent itself, are relatively insensitive to temperature and pressure and are conveniently performed at ambient or room temperature (i.e., 22.5 • C +/- 2.5 • C) and atmospheric pressure.

Lipids used in this invention are obtainable from a number of sources. Natural phosphatide mixtures from egg or soy containing more than 70% phosphatidylcholine are obtained from a number of commercial sources such as Sigma Chemical of St. Louis, MO, and Lipoid KG, Ludwigshafen, West Ger., Hepar of Franklin, Ohio. Hepar supplies egg phosphatidylcholine. Other sources of lipid such as soy phosphatidylcholine are American Lecithin, Woodside, L.I., NY, and Riceland Foods, Little Rock, Arkansas. Phosphatidic acid of 99% purity is obtained from Avanti Chemical of Birmingham, AL.

A method for desalting utilizes trichlorofluromethane (CCl_3F) (E.I. du Pont de Nemours & Co., Wilmington, Del., under the trademark Freon 11). In this method phosphatides are added to a mixture of absolute ethanol and CCl_3F at a ratio of from 0.5:1 to 1:0.5 with 1:1 being most preferred to form a solution.

A temperature of 15 • C is preferred at atmospheric pressure but any temperature below the boiling point of CCl_3F at the operating pressure is suitable, particularly 20-35 • C under pressure.

About 5 g of phosphatide may be added to about 40 ml of the $CC1_3F$/ethanol mixture but this proportion may be increased and is limited only by the formation of emulsion upon making of two phases. Up to a 20 wt % mixture of phosphatide:CCl_3F/ethanol solvent may be used with 1-10 wt % being preferred. The resulting solution is titrated with a slight excess of dilute acid such as HCl and the solution is mixed by any convenient method including stirring, shaking and sonication. The slight emulsion formed is permitted to separate and usually this requires only a matter of minutes.

The CCl_3F layer is removed and an ethanol:water (about 2:1 - 1:2 ethanol water v/v) mixture is then added to the CCl_3F solution for repeated washing and removal of excess acid, until the upper phase is neutral. The lower CCl_3F solution is then warmed to about room temperature (i.e., 22.5 • C +/- 2.5 • C) to evaporate the CCl_3F leaving the desalted lipid residue. Care is required in warming so that frothing does not occur. The solvent is then removed. This is conveniently accomplished first by evaporation with a thin stream of nitrogen at 22.5 • C +/- 2.5 • C and then by rotoevaporation. Again care is taken so that frothing/bumping does not occur.

The lipid solvents of this invention must be (1) dissolving of lipids, (2) substantially soluble (termed herein "miscible") in water, and (3) pharmaceutically acceptable. As the lipid solvent will only appear in the administered dose upon dilution by the aqueous phase and the dilution would ordinarily constituted a 5 to 50 times reduction in lipid solvent concentration a number of pharmaceutically acceptable lipid solvents are available. These include ethanol, polyethylene glycol and propylene glycol. The preferred polyethylene glycols have molecular weights of 400 to 800 with 800 most preferred. Absolute ethanol is the preferred lipid solvent, but any pharmaceutically acceptable lipid solvent may be used.

The lipid solvent must be miscible or at least significantly soluble with the aqueous solution in order to deliver simultaneously both the pharmacological agent and lipid to this solution as well as for the purpose of diluting the lipid solvent in the aqueous solution.

The lipid solvents for the solution preparation must be substantially water free but water miscible. The presence of excess water in the preparation will cause the lipophilic pharmacological agents to be insoluble and can adversely affect the storage stability of the preparation through hydrolysis. The maximum amount of water will vary with the specific agent but generally will be less than 1% and not greater than 5% or 10% (w/w). In practice the maximum amount of water tolerable in a system is easily determined in that if excess water is present the solution becomes cloudy indicating the presence of precipitate or liposomes. Certain pharmacological agents susceptable to hydrolysis such as salicylic acid acetate do not tolerate the presence of water even at about 5% in ethanol, though lesser amounts of water are tolerable in this system. Substantially water-free lipid solvents wherein the pharmacological agent-lipid is not appreciably hydrolized or rendered insoluble will be termed nonaqueous.

Due to the high flammability of absolute ethanol, admixing with at least 10% lipid solvent diluent such as polyethylene glycol 400 or 800 is preferred and up to 30% polyethylene glycol 800 most preferred in reducing flammability of the preparation while maintaining pharmaceutical acceptability. Other weights of polyethylen glycol and other lipid solvent diluents are also acceptable.

Sterility of drug-lipid solution is necessary both for a prolonged shelf life as well as subsequent use of this solution. Therefore, drug-lipid solution is conveniently terminally sterilized by filtration. This is preferably done through a 0.2 µm polycarbonate filter, cellulose-containing filter or other inert filter that does not interact either with lipid solvent or with the solubilized drug or lipid. Filtration also removes undissolved particles from the preparation. Sterilization by filtration is a particular advantage of this preparation.

Storage stability of the pharmocological agent-lipid solution preparation will vary but is directly related to the stability of the lipids. The storage stability is enhanced by storing at reduced temperatures.

The pharmacological agent-lipid solution may be advantageously employed by direct administration wherein a suspension will be formed in vivo wherein the aqueous medium is the physiological environment. In such application a pharmacological agent-lipid solution, such as indomethacin mixed with a non-aqueous water-miscible lipid solvent such as ethanol and a desalted charged lipid, when ingested, conveniently in capsule or liquid form, becomes liposomal in the gastric environment. Other suitable oral dosage forms are dragees, troches, lozenges, tablets and additional forms known to those skilled in the art. Oral dosage forms configured and adapted for oral administration to subjects in need of such dosages shall be termed unit oral dosage forms.

Aggregate suspensions prepared from the pharmacological agent-lipid solution preparation are made by agitating the preparation in an aqueous medium. Agitation is accomplished by any convenient method, but is most easily accomplished by loading the solution preparation into a syringe and then injecting the preparation into the aqueous medium as contained within an ampoule or container. The exact rate of

injection is not critical. Injection of the solution preparation should be accompanied by rapid mixing such that the water miscible lipid solvent and the aqueousmedium rapidly mix. Beyond the syringe method other convenient methods of preparing the suspension are pouring, dropping, or spraying in while hand mixing, vortexing, stirring or sonicating.

Any pharmaceutically acceptable aqueous medium may be used. Examples of suitable aqueous media are water, 5% dextrose in water, physiological citrate buffer, physiological phosphate buffer and 0.9% saline. As used here in referring to physiological buffers indicates pharmaceutical acceptability in view of the intended use in animals such as mammmals (including humans). The use of such medium will be both for formation of the suspension and as a pharmaceutical diluent. For oral administration the preferred aqueous medium is water or palatable fluids such as fruit juices and syrups or infusions such as teas and coffee.

At moderate pharmacological agent to lipid ratios pharmacological agent-lipid solution will generally form aggregates in the structure of liposomes upon mixing with aqueous medium.

At higher pharmacological agent to lipid ratios, the aggregate structure becomes nonliposomal. For example, at pharmacological agent to lipid ratios of 1:1 (wt/wt), the aggregates are spherical particles of about 0.2um or higher, have minimal entrapped water, appear to be temporarily closely associated with the water miscible lipid solvent, and upon centrifugation appear denser than liposomes of similar pharmacological agent and lipid composition Higher pharmacological agent to lipid ratios will be understood to mean from about 20 moles percent pharmacological agent to lipid ratio up to 60 moles percent or more.

The formation of aggregates upon formation of suspension is strongly related to the pharmacological agent/lipid ratio (wt/wt) of the solution preparations. Examination of this ratio was done from 10:1 to 0.5:1. In general, aggregate diameter and suspension opacity decreased as less lipid in relation to pharmacological agent was utilized.

At the higher pharmacological agent to lipid ratios, the aggregates were of submicron size and the suspensions colloidal, thus the physical parameters of the suspenson are adjustable by varying the ratios of the pharmacological agent to lipid.

Preparations of pharmacological agent-lipid solution such as those with cyclosporin A used in this invention are preferably begun by the dissolving of the agent and/or the lipid into lipid solvent. While this can conveniently be accomplished at 22.5 • C +/- 2.5 • C, using a heated water bath, facilitates dissolving. For cyclosporin A, a water bath at 40 • C - 50 • C facilitates the dissolution.

Generally, lipids and pharmacological agent are separately solubilized into lipid solvent as stock solutions at convenient concentrations. Stock solutions can be maintained at convenient and nondegrading temperature, for example 4 • C. When preparing a particular pharmacological agent-lipid solution preparation of the present invention appropriate aliquots of stock solutions were combined to achieve desired final concentrations of lipid and agent.

However, it is quite acceptable to add pharmacological agent and lipid directly to the lipid solvent of a preparation. In such circumstance it is preferable to add the lipid to the lipid solvent first as the lipid in some circumstances increases the solubility of a pharmacological agent. This co-solubilizing or "salting-in" may be up to a 50% increase in solubility or apparent solubility with agents such as salicylic acid acetate and indomethacin. In the context of salting in the term "apparent solubility" recognizes that a pharmacological agent salted in may be in the form of a complex with dissolved lipid rather than truly solubilized, such as is the case with amphotericin B.

The co-solubilizing or salting in is a suprising aspect of this invention as to pharmacological agents that will associate with lipid. Lipid soluble pharmacological agents include the nonsteroidal anti-inflammatories such as salicylic acid acetate and indomethacin as noted above. To practice this aspect of the invention requires dissolving lipid in the lipid solvent, such as ethanol, forming a co-solution prior to addition of the pharmacological agent to be co-solubilized. From 10% lipid by weight up to the solubility limit of the particular lipid (or lipids) in the lipid solvent may be used as the solution in which to co-solubilize the lipid soluble (or apparently soluble) pharmacological agent.

After the solution of lipid and lipid solvent is made diluents such as polyethylene glycol (which may also be a lipid solvent) can be added to reduce flammability. Antioxidants may also be added then. The final pharmacological agent-lipid solution preparation is conveniently stored in an ampoule and preferably at 4 • C.

The pharmacological agent-lipid solution preparation concentration of constituents will only be limited by the relative solubility of such constituents with a view to the desired final concentration. A typical preparation will be comprised of up to 0.5 gm of drug/ml of lipid solvent with 0.5 gm of lipid.

In the preferred pharmacological agent-lipid solution preparation storage stability is enhanced by the inclusion of an antioxidant such as alpha-tocopherol. This is present in amounts at 0.1 to 1% (wt/wt) relative

to the amount of lipid.

To determine whether phospholipids can increase the solubility or apparent solubility of a pharmacological agent such as salicylic acid acetate, both drug and lipid were co-solubilized in a lipid solvent such as absolute ethanol (USP). Five ml of this lipid solvent was able to dissolve about a maximum of 1 gr. salicylic acid acetate. A lipid such as egg phosphatidylcholine was completely dissolved in 5 ml of ethanol in a series of test tubes and to this solution crystals of the test pharmacological agent salicylic acid acetate were added gradually and dissolved. The maximum amount of salicylic acid acetate dissolved in 5 ml ethanol containing 1.5 gm egg phosphatidylcholine was 1.5 gm indicating a 50% increase in solubility of drug under these conditions. Any convenient temperature and pressur may be used for this procedure that will dot adversely affect the pharmacological agent or boil the lipid solvent.

This "salting in" illustrates for one skilled in the art the use of lipids for increasing the solubility of lipid soluble drugs up to 50% or more. Salicylic acid acetate solubility in an egg phosphatidylcholine ethanol is seen to increase about 50% and the solubility off indomethacin in a similar system is seen to increase 50%. Amphoteracin B is similarly salted in but appears to do so in the form of complexes with lipid rather than simple solubilizing.

A number of analytical steps known in the art are useful in selecting those lipid/lipid solvent systems which upon mixture with an aqueous medium, generate pharmaceutically acceptable suspensions. Such analytical steps include assessment by visual inspection for appearance, opacity and the presence of crystals, precipitates or sediment; (b) light microscopic examination such as in a Neubauer cytometer with a micrometer scale at 100 times and 400 times magnification; (c) turbidimetric measurements by assessing transmission at 520 nm; (d) electron microscopy of negatively stained preparations; (e) quasielastic light scattering (QELS) for determination of mean particle dimension; (f) ultracentrifugation; (g) organ distribution after intravenous innoculation of aggregate suspension having aggregates labeled with a reporting group such as a radioactive tracer, (e.g., ^{3}H-cyclosporin when using cyclosporin); and (h) bioactivity in cell culture of a particular cell type (e.g., for cyclosporin, spleen lymphocytes stimulated with concanavaline A and labeled with ^{3}H-thymidine).

In addition, such suspensions must be without large aggregations, precipitates or crystals. The suspension must remain free off large aggregations precipitates or crystals during the time necessary for preparing and administering injections under hospital conditions. This time was presumed to be at minimum from about 15 minutes to about two hours. For the suspensions intended for intravenous administration, selection for small aggregate size is necessary as suspensions containing bodies, such as aggregates, over 5 µm in diameter are not usually suitable for intravenous administration. Thus, those skilled in the art may rapidly define a lipid solvent system suspension suitable for internal and particularly intravenous administration.

The results of organ distribution of aggregates showed that the aggregates do not accumulate in liver and spleen as would be predicted for liposomes.

The pharmacological agent-lipid solution preparations of this invention are useful for treating animals (including humans) in need of such treatment. Treatment as used herein includes administration of any pharmacological agents such as diagnostic materials, biologically active agents and contrast materials.

Treatment is frequently accomplished by preparing a suspension from the solution preparation and administering the suspension in therapeutically effective amounts. However, as noted, the pharmacological agent-lipid solution may be directly administered for treating mammals. A therapeutically effective amount will be understood to mean a sufficient amount to achieve a physical or physiological response, and for known drugs will generally be the same dose for the existing dosage forms of the drug.

The therapeutically effective amount of a given pharmacological agent will vary with the purpose of the administration, the particularities of the recipient and other factors well known in the art.

In a liposome-drug delivery system, a pharmacological agent such as a drug is entrapped in or associated with the liposome and then administered to the patient to be treated. For example, see Rahman et al., U.S. Patent No. 3,993,754, Sears, U.S. Patent No. 4,145,410; Paphadjopoulos et al., U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,114,179; Lenk et al., U.S. Patent No. 4,522,803; and Fountain et al., U.S. Patent No. 4,588,578.

The mode of administration of the preparation may determine the sites and cells in the organism to which the compound will be delivered. Aggergates of this invention can be administered alone but will generally be administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. The preparations may be injected parenterally, for example, intra-arterically or intravenously. The preparations may also be administered via oral, subcutaneous, or intramuscular routes, or by inhalation. For parenteral administration, they can be used, for example, in the form of a sterile aqueous solution which may contain other solutes, for example, enough

salts or glucose to make the solution isotonic. Other uses, depending upon the particular properties of the preparation, may be envisioned by those skilled in the art.

For administration to humans in the curative treatment of disease states, the prescribing physician will ultimately determine the appropriate dosage for a given human subject, and this can be expected to vary according to the age, weight, and response of the individual as well as the nature and severity of the patient's disease. The dosage of the drug in aggregate form will generally be about that.employed for the free drug. In some cases, however, it may be necessary to administer doses outside these limits.

This invention will be better understood by reference to the following examples which are merely illustrative of the invention.

EXAMPLE 1

Cyclosporin A Solution Preparation:Desalted Lipids

Stock solution of cyclosporin A (Sandoz Pharmaceuticals Corporation, East Hanover, NJ) at 200 mg/ml and stock solution of desalted egg phosphatides (Hepar Industrial, Inc., Franklin, Ohio) at 250mg/ml were prepared in advance and kept at 4 °C. To prepare 10 ml of cyclosporin-lipid solution 2 ml of lipid stock solution was brought up to 8.75 ml with absolute ethanol and mixed by hand at 22.5 °C +/- 2.5 °C and atmospheric pressure. To this solution 1.25 ml of cyclosporin A stock was added and mixed again The final solution containing cyclosporin 25 mg/ml and desalted egg phosphatides 50 mg/ml was filtered through 0.2 µm polycarbonate filter. The solution was then bubbled through with oxygen free nitrogen for 10 seconds, overlayed with nitrogen and tightly enclosed.

EXAMPLE 2

Cyclosporin A Solution Preparation:Desalted and Neutral Lipid s

1.25 gm of dry powdered egg yolk phosphatides (nondesalted) (Hepar) were dissolved in 2 ml absolute ethanol by heating at 56 °C for 10 min. in a water bath. The solution was then cooled in a ice basket and filtered through a 1.0 micron polycarbonate filter (Nucleopore, Pleasanton, CA). The resulting lipid solution was adjusted to a concentration of 400 mg lipids/ml with absolute ethanol. Desalted phosphatidic acid was dissolved in ethanol at a concentration of 200 mg/ml. 0.63 ml of the egg yolk phosphatide solution was mixed with 0.63 ml of a cyclosporin A-ethanol solution containing 200 mg cyclosporin/ml drug to which was then added 0.06 ml of desalted phosphatidic acid ethanol solution followed by mixing. All mixing steps took only a matter of minutes. The resulting mixture was adjusted to 5 ml with absolute ethanol to a final concentration of 50 mg egg phosphatides, 6.25 mg desalted phosphatidic acid and 25 mg cyclosporin A per ml ethanol. This solution was sterilized by filtration through 0.2 µm polycarbonate filter (Nucleopore, Pleasanton, CA), bubbled through with oxygen-free nitrogen and sealed in an ampoule.

EXAMPLE 3

Cyclosporin A Solution Preparation and Suspension

Soy phosphatidylcholine after removal of absolute ethanol insoluble impurities and being desalted was dissolved in absolute ethanol at 22.5 °C +/- 2.5 °C and atmospheric pressure at a concentration of 382 mg/ml. Cyclosporin A was dissolved in a separate aliquot of absolute ethanol at a concentration of 200 mg/ml.

The cyclosporin A-lipid solution was prepared by adding sequentially in a glass tube 0.327 ml lipid solution (125 mg), 1.25 ml cyclosporin A Solution (250 mg), absolute ethanol 1.87 ml (1.47 gm) and 6.50 ml (7.34 gm) polyethylene glycol 400

After each addition solutions were briefly shaken and the final solution was vortexed. This solution contained 50 mg cyclosporin A, and 25mg lipid in 1 ml of 65% polyethylene glycol 400 in absolute ethanol.

A suspension was formed by adding 20 mg of this solution by injection into 500 ml 5% dextrose in water. The aggregates in the suspension thus formed were 1.0 µm or smaller.

The aggregates including the aggregates in suspension were assessed by: (a) visual inspection for appearance, opacity and presence of concretions, crystals, precipitates or sediment; (b) light microscopic examination in a Neubauer cytometer with a micrometer scale at 100 times and 400 times magnification; (c) turbidimetric measurements by assessing transmission at 520 nm; (d) electron microscopy of negatively

stained preparations; (e) quasielastic light scattering (QELS) for determination of mean particle dimension; (f) ultracentrifugation; (g) organ distribution after intravenous innoculation of aggregate suspension having aggregates labeled with ^{3}H-cyclosporin; and (h) bioactivity in cell culture of spleen lymphocytes stimulated with concanavaline A and labeled with ^{3}H-thymidine.

QELS analysis indicated that the mean diameter of aggregates was below 0.3 µm (Table 1). Turbidimetric measurements of suspensions kept at 22.5°C+/-2.5°C without agitation showed that the transmission at 520 nm gave similar value at "zero" time (shortly after suspension was formed) and at subsequent time points (Fig. 1) indicating a rapidly forming and stable suspension.

Electron microscopy revealed round droplet-like particles having a diameter below 1.0 µm in agreement with light microscopy examination and QELS results. All three measurements clearly showed that the dimension of aggregates can be modulated by changing the pharmaceutical agent/lipid ratio and the nature of the lipids used.

Ultracentrifugation analysis showed that the aggregates sediment when the suspension is placed over a Hypaque-Ficoll solution diluted one-third with distilled water and having a density higher than 1.0. Under the same preparation and centrifugation conditions, liposomes formed by dispersion of lipids alone in aqueous medium, do not sediment.

EXAMPLE 4

Cyclosporin A Suspension

A sample for parenteral administration from the solution of Example 1 was prepared by taking the content of an ampuole (10 ml ethanol containing 500 mg egg phosphatides which were desalted and 250 mg cyclosporin A with a #23G needle (1.5 in) adapted to a 10 ml syringe. The needle was inserted through the rubber stopper of a 500 ml bottle containing 5% dextrose in water. The bottle was kept upside down and mixed by hand to create a vortex, while the contents of the syringe was infused over 20 seconds continuously into the aqueous solution. After injection was completed, the needle was retracted and the bottle shaken by hand for 10 sec. The resulting aggregates, the cyclosporin A-lipid suspension, was allowed to stand for 15 minutes to allow disappearance of the gas bubbles formed during shaking. This suspension was intended for use within 15 min. to 6 hrs after preparation.

EXAMPLE 5

Cyclosporin Behavior in vivo

Cyclosporin A aggregates were slightly more efficacious in suppressing lymphocyte proliferation as measured by ^{3}H-thymidine uptake, than the cyclosporin solubilized in polyethoxylated castor oil and ethanol.

The dimensions of the nonliposomal lipid aggregates wherein the pharmacological agent is cyclosporin A are relatively uniform of a size 250 nm +/- 20 nm.

The results of organ distribution of aggregates showed that the aggregates do not accumulate in liver and spleen as would be predicted for liposomes.

EXAMPLE 6

Aspirin Solution Preparation

Desalted egg phosphatides (Hepar) were dissolved in ethanol at a concentration of 250 mg/ml in a 1000 ml flask. To 400 mg of salicylic acid acetate, 1.6 ml of the above solution containing 400 mg of phosphatides was added and the solution adjusted with absolute ethanol to 4 ml. The final concentration of both ingredients in this pharmacological agent-lipid solution was 100 mg/ml. This procedure was performed at 22.5°C+/- 2.5°C and atmospheric pressure.

EXAMPLE 7

Aspirin Suspension

To prepare a suspension, desalted egg phosphatides (Hepar) were dissolved in ethanol at a concentration of 250 mg/ml in a 1000 ml flask. To 400 mg of salicylic acid acetate, 1.6 ml of the above solution containing 400 mg of phosphatides was added and the solution adjusted with absolute ethanol to 4 ml. The final concentration of both ingredients in this pharmacological agent-lipid solution was 100 mg/ml. This procedure was performed at 22.5 • C +/-2.5 • C and atmospheric pressure. Then 0.5 ml of the salicylic acid acetate-lipid solution was added to 9.5 ml of distilled water and briefly shaken by hand, at 22.5 • C +/- 2.5 • C and atmospheric pressure forming a suspension. The resulting suspension was milky in appearance and did not contain visable crystals or aggregates after a 30 minute period. Light microscopy of the suspension revealed aggregates, primarily with a diameter below 5 μm and no crystal characteristic for salicylic acid acetate. This suspension contained 0.5 mg salicylic acid acetate/ml.

EXAMPLE 8

Topical/Oral Aspirin Suspension

A dosage form of salicylic acid acetate for topical or oral use was prepared as follows: Desalted egg phosphatides (Hepar) were dissolved in absolute ethanol at a concentration of 250 mg/ml in a 1000 ml flask. To 400 mg of salicylic acid acetate, 1.6 ml of the above solution containing 400 mg of phosphatides was added and the solution adjusted with absolute ethanol to 4 ml. The final concentration of both ingredients in this pharmacological agent-lipid solution was 100 mg/ml. This procedure was performed at 22.5 • C +/- 2.5 • C and atmospheric pressure.
Next, the topical/oral administration dosage form was prepared by adding the pharmacological agent-lipid solution, to water at 22.5 • C +/- 2.5 • C and briefly agitating the mixture. A cloudy suspension promptly formed. This salicylic acid acetate formulation may then be ingested or used topically.

EXAMPLE 9

Indomethacin Preparation

15g of egg phosphatides (Lipoid E80, Lipoid KG, Ludwigshafen, West Ger.) containing 80% phosphatidylcholine was solubilized in 3ml absolute ethanol. The resulting co-mixture then solublized indomethacin, 1g of which was then added to the co-mixture. The final preparation contained 25mg of indomethacin and 375mg of lipid per 0.4ml. This precedure was performed at 22.5 • C +/-2.5 • C at and atmospheric pressure. The ethanol concentration of the preparation was 0.075ml/0.4ml. The preparation was encapsulated in a soft gelatin capsule as a unit oral dosage form.

PREPARATION 1

Desalting of Lipids: Hexane-Ethanol-Hydrochloric Acid Procedure

Natural phosphatide mixtures from soy containing more than 70% phosphatidylcholine (PC) were dissolved in hexane at 1 gm lipid/10 ml solvent. To this solution 6.6 ml of absolute ethanol and 3.3 ml of 0.2N HCl was added and mixed thoroughly at atmospheric pressure and at 22.5 • C +/-2.5 • C. Phases formed and were permitted to separate and the lower aqueous phase discarded. The hexane phase was repeatedly washed with ethanol-water, 1:1 (v/v) until the pH in the lower aqueous phase was neutral. The resulting desalted lipids were recovered from the hexane phase by removal of the hexane by rotoevaporation at 35 • C and 100mm Hg.

PREPARATION 2

Desalting of Lipids:CCl_3F

At atmospheric pressure and in a cold room (4°-10°C) 5 grams of egg phosphatides (Hepar) were dissolved in a mixture of absolute ethanol:CCl_3F (Freon 11, du Pont), 1:1 (40 ml) at 15 • C; 25 ml of 0.5 N

aqueous HCl was added and the mixture shaken The lower CCl_3F phase was removed after the emulsion was broken and mixed with 20 ml of absolute ethanol and 25 ml of water. The CCl_3F lower phase was again removed and the ethanol/water wash was repeated until the upper aqueous phase was neutral. The lower CCl_3F solution was allowed to warm to 22.5 °C +/- 2.5 °C and the solvent driven off with a stream of nitrogen and finally, on a rotoevaporator. Yield = 4.9 grams of desalted lipids.

PREPARATION 3

Desalted Lipids:Ionic Exchange Resin

20 gm of egg phosphatides (Hepar) dissolved in 100 ml of absolute ethanol was passed through 200 g of the cation exchange resin (Biorad of Richmond, VA) (AG 50 WX8) in the hydrogen form and in ethanol. The column was further diluted with 50 ml of ethanol, at 22.5 °C +/-2.5 °C and atmospheric pressure. The total eluant of the first volume was passed through 200 g column of anion exchange resin (Biorad AG1-X8) in the hydroxyl form and in ethanol. The columns were washed with 50 ml of absolute ethanol and the total 200 ml of eluant contained desalted phospholipids at a concentration of 10 g/100 ml of ethanol. This was useable directly or diluted further with ethanol.

PREPARATION 4

Salting In

Five ml of absolute ethanol was able to dissolve maximum 1 gr. salicylic acid acetate. Egg phosphatidylcholine (Hepar) 1.5 gm was completely dissolved in 5 ml of ethanol in a series of test tubes and to this solution crystals of salicylic acid acetate were added gradually and dissolved. The maximum amount of salicylic acid acetate dissolved in 5 ml ethanol containing 1.5 gm egg phosphatidylcholine was 1.5 gm indicating a 50% increase in solubility of drug. To accelerate the dissolving process all test tubes containing crystals of salicylic acid acetate and lipid solvent were agitated gently in a water bath of 40 °C and cooled to 22.5 °C +/- 2.5 °C after crystals were completely dissolved.

Table 1

QELS ANALYSIS OF AGGREGATE DIMENSION		
	MEAN DIAMETER (nm)	
SAMPLE	NICOMP ANALYSIS*	GAUSSIAN ANALYSIS
HDrrC4-33	129.0	150.0
HDrrC5-34	183.00	144.0
HDrrC6-35	258.00	176.7
HDrrC7-36	209.00	143.4
JDrrC1-37	166.0	151.4
$\bar{X} \pm S$:	189.0 ± 48	153.0 ± 13

*NICOMP analysis is a data reduction extracting the component sizes contributing to the exponential curve.

Claims

1. A pharmacological agent-lipid solution preparation comprising
   (a) a desalted charged lipid
   (b) a nonaqueous water-miscible lipid solvent
   (c) a lipid soluble pharmacological agent :
   and preferably in unit dosage form.

2. The preparation of claim 1 wherein the desalted charged lipid is phosphatidic acid, dicetylphosphate, phosphatidylethanolamine or phosphatidylserine.

3. The preparation of anyone of claims 1 to 2 wherein the solvent comprises at least 10% (wt/wt) polyethylene glycol of molecular weight of from to 400 to 800 and optionally 30% polyethylene glycol of molecular weight of from 400 to 800 (wt/wt).

4. The preparation of anyone of claims 1 to 3 wherein the solvent comprises absolute ethanol.

5. The preparation of anyone of claims 1 to 4 wherein the pharmacological agent is an immunomodulator, an antifungal, antiinflammatory, antineoplastic agent or hormone.

6. The preparation of claim 5 wherein said agent is a polypeptide having a molecular weight of greater than 1000.

7. The preparation of claim 5 wherein the pharmacological agent is an immunomodulator, preferably cyclosporin A.

8. The preparation of claim 5 wherein the pharmacological agent is an antifungal agent, preferably miconasole, terconazole or amphotericin B.

9. The preparation of claim 5 wherein the pharmacological agent is an anti-inflammatory, preferably prednisone, dexamethasone, fluoromethasone, indomethacin, salicylic acid and acetate or ibuprofen and preferably in a unit oral dosage form.

10. The preparation of claim 9 additionally comprising at least 70% by weight phosphatidylcholine.

11. The preparation of claims 9 or 10 wherein indomethacin is present from 0.5% to 30% by weight or preferably present at from 10 to 20% by weight and preferably in unit dosage form.

12. The preparation of claim 11 additionally comprising at least 70% by weight phosphatidylcholine in unit dosage form.

13. The preparation of claim 5 wherein the pharmacological agent is an antineoplastic preferably doxorubicin.

14. The preparation of claim 5 wherein the pharmacological agent is a hormone preferably a glucocorticoid, mineralocorticoid or estrogen.

15. The preparation of anyone of claims 1 to 14 further comprising (d) a pharmaceutically acceptable aqueous medium.

16. A pharmacological preparation according to claim 1 comprising absolute ethanol, lipid, and nonsteroidal anti-inflammatory, preferably wherein the nonsteroidal anti-inflammatory is indomethacin and preferably wherein the indomethacin is present from 0.5% to 30% by weight or form 10 to 20% by weight.

17. A method of preparing a suspension from the preparation of claims 1 to 15 comprising adding said preparation to a pharmaceutically acceptable aqueous medium, optionally at a ratio of at least 0.1:1 v/v and preferably wherein the aqueous medium is water, 5% dextrose in water, 0.9% saline, physiological phosphate buffer, or physiological citrate buffer.

18. The method of claim 16 wherein said suspension contains aggregates in which the concentration of pharmacological agent to lipid is at least 20 moles percent.

19. The process of preparing a pharmacological agent-lipid solution preparation according to anyone of claims 1 to 15 comprising
(a) a desalted charged lipid
(b) a nonaqueous water-miscible lipid solvent, and
(c) a lipid soluble pharmacological agent ;
wherein the process comprises the step of admixing (a), (b), and (c).

20. The process of claim 19 additionally admixing at least about 70% by weight phosphatidylcholine.

21. The process of claims 19 or 20 further comprising combining (d) a pharmaceutically acceptable aqueous medium.

22. The process of combining into a pharmacological preparation absolute ethanol, lipid, and nonsteroidal anti-inflammatory, according to claim 19 wherein the nonsteroidal anti-inflammatory is indomethacin, and preferably wherein the indomethacin is present from 0.5% to about 30% by weight or from 10 to 20% by weight.

Patentansprüche

1. Zubereitung einer pharmakologischen Wirkstoff-Lipid-Lösung, enthaltend:
   (a) ein entsalztes, geladenes Lipid,
   (b) ein nicht-wäßriges mit Wasser mischbares Lipid-Lösungsmittel,
   (c) einen Lipid-löslichen pharmakologischen Wirkstoff;
   und vorzugsweise in Dosiseinheits-Form.

2. Zubereitung nach Anspruch 1, worin das entsalzte geladene Lipid Phosphatidsäure, Dicetylphosphat, Phosphatidyl-ethanolamin oder Phosphatidyl-serin ist.

3. Zubereitung nach einem der Ansprüche 1 bis 2, worin das Lösungsmittel mindestens 10 % (Gew/Gew) Polyethylenglykol mit einem Molekulargewicht von 400 bis 800 und gegebenenfalls 30 % Polyethylenglykol mit einem Molekulargewicht von 400 bis 800 umfaßt (Gew/Gew).

4. Zubereitung nach einem der Ansprüche 1 bis 3, worin das Lösungsmittel absolutes Ethanol umfaßt.

5. Zubereitung nach einem der Ansprüche 1 bis 4, worin der pharmakologische Wirkstoff ein Immunmodulator, ein Antifungusmittel, ein antiinflammatorisches Mittel, ein antineoplastisches Mittel oder Hormon ist.

6. Zubereitung nach Anspruch 5, worin der Wirkstoff ein Polypeptide mit einem Molekulargewicht von über 1000 ist.

7. Zubereitung nach Anspruch 5, worin der pharmakologische Wirkstoff ein Immunmodulator, vorzugsweise Cyclosporin A, ist.

8. Zubereitung nach Anspruch 5, worin der pharmakologische Wirkstoff ein Antifungusmittel, vorzugsweise Miconazol, Terconazol oder Amphotericin B ist.

9. Zubereitung nach Anspruch 5, worin der pharmakologische Wirkstoff ein antiinflammatorisches Mittel ist, vorzugsweise Prednison, Dexamethason, Fluoromethason, Indomethacin, Salicylsäure und Acetat oder Ibuprofen, und vorzugsweise in einer oralen Dosiseinheits-Form.

10. Zubereitung nach Anspruch 9, enthaltend zusätzlich mindestens 70 Gew.-% Phosphatidylcholin.

11. Zubereitung nach den Ansprüchen 9 oder 10, worin Indomethacin von 0,5 Gew.-% bis 30 Gew.-% oder vorzugsweise von 10 bis 20 Gew.-% vorhanden ist, und vorzugsweise in Dosiseinheits-Form.

12. Zubereitung nach Anspruch 11, enthaltend zusätzlich mindestens 70 Gew.-% Phosphatidylcholin, in Dosiseinheits-Form.

13. Zubereitung nach Anspruch 5, worin der pharmakologische Wirkstoff ein antineoplastisches Mittel, vorzugsweise Doxorubicin, ist.

14. Zubereitung nach Anspruch 5, worin der pharmakologische Wirkstoff ein Hormon, vorzugsweise ein Glucocorticoid, Mineralocorticoid oder Östrogen, ist.

15. Zubereitung nach einem der Ansprüche 1 bis 14, enthaltend darüber hinaus (d) ein pharmazeutisch brauchbares wäßriges Medium.

16. Pharmakologische Zubereitung nach Anspruch 1, enthaltend absolutes Ethanol, Lipid und nichtsteorides antiinflammatorisches Mittel, worin vorzugsweise das nichtsteroide antiinflammatorische Mittel Indomethacin ist und worin vorzugsweise das Indomethacin von 0,5 Gew.-% bis 30 Gew.-% oder von 10 bis 20 Gew.-% vorhanden ist.

17. Verfahren zur Herstellung einer Suspension aus der Zubereitung der Ansprüche 1 bis 15, umfassend den Zusatz dieser Zubereitung zu einem pharmazeutisch brauchbaren, wäßrigen Medium, gegebenenfalls in einem Verhältnis von mindestens 0,1 : 1 Vol/Vol und wobei vorzugsweise das wäßrige Medium Wasser, 5 % Dextrose in Wasser, 0,9 % Salzlösung, physiologischer Phosphatpuffer oder physiologischer Citratpuffer ist.

18. Verfahren nach Anspruch 16, worin die Suspension Aggregate enthält, in denen die Konzentration von pharmakologischem Wirkstoff zu Lipid mindestens 20 Molprozent ist.

19. Verfahren zur Herstellung einer Zubereitung einer pharmakologischen Wirkstoff-Lipid-Lösung gemäß einem der Ansprüche 1 bis 15, enthaltend
(a) ein entsalztes, geladenes Lipid,
(b) ein nicht-wäßriges, mit Wasser mischbares Lipid-Lösungsmittel, und
(c) einen Lipid-löslichen pharmakologischen Wirkstoff;
wobei das Verfahren die Stufen des Vermischens von (a), (b) und (c) umfaßt.

20. Verfahren nach Anspruch 19, wobei zusätzlich mindestens etwa 70 Gew.-% Phosphatidylcholin zugemischt werden.

21. Verfahren nach Anspruch 19 oder 20, wobei darüber hinaus (d) mit einem pharmazeutisch brauchbaren wäßrigen Medium kombiniert wird.

22. Verfahren zum Einbringen in eine pharmakologische Zubereitung von absolutem Ethanol, Lipid und nichtsteroidem, antiinflammatorischem Mittel, nach Anspruch 19, wobei das nichtsteroide, antiinflammatorische Mittel Indomethacin ist und wobei vorzugsweise das Indomethacin von 0,5 Gew.-% bis etwa 30 Gew.-% oder von 10 bis 20 Gew.-% vorhanden ist.

Revendications

1. Composition en solution de lipide-agent pharmacologique comprenant
(a) un lipide chargé privé de sels,
(b) un solvant pour lipides, non aqueux, miscible à l'eau,
(c) un agent pharmacologique lipososuble,
et de préférence sous forme de dose unitaire.

2. Composition selon la revendication 1, dans laquelle le lipide chargé privé de sels est un acide phosphatidique, le phosphate de diacétyle, la phosphatidyléthanolamine ou la phophatidylsérine.

3. Composition selon la revendication 1 ou 2, dans laquelle le solvant comprend au moins 10 % (p/p) d'un polyéthylèneglycol ayant une masse moléculaire de 400 à 800, de préférence 30 % (p/p) d'un polyéthylèneglycol ayant une masse moléculaire de 400 à 800.

4. Composition selon l'une quelconque des revendications 1 à 3, dans laquelle le solvant comprend de l'éthanol absolu.

5. Composition selon l'une quelconque des revendications 1 à 4, dans laquelle l'agent pharmacologique est un agent immunomodulateur, antifongique, anti-inflammatoire, antinéoplasique ou une hormone.

6. Composition selon la revendication 5, dans laquelle ledit agent est un polypeptide ayant une masse moléculaire de plus de 1 000.

7. Composition selon la revendication 5, dans laquelle l'agent pharmacologique est un immunomodulateur, de préférence la cyclosporine A.

8. Composition selon la revendication 5, dans laquelle l'agent pharmacologique est un agent antifongique, de préférence le miconazole, le terconazole ou l'amphotéricine B.

9. Composition selon la revendication 5, dans laquelle l'agent pharmacologique est un anti-inflammatoire, de préférence la prednisone, la dexaméthasone, la fluorométhasone, l'indométhacine, l'acide salicylique et son acétate ou l'ibuprofène, et de préférence sous forme de dose unitaire.

10. Composition selon la revendication 9, comprenant au moins 70 % en poids de phosphatidylcholine.

11. Composition selon la revendication 9 ou 10, dans laquelle de l'indométhacine est présente à raison de 0,5 à 30 % en poids, ou de préférence est présente à raison de 10 à 20 % en poids, et de préférence sous forme de dose unitaire.

12. Composition selon la revendication 1, comprenant en outre au moins 70 % en poids de phosphatidylcholine sous forme de dose unitaire.

13. Composition selon la revendication 5, dans laquelle l'agent pharmacologique est un agent antinéoplasique, de préférence la doxorubicine.

14. Composition selon la revendication 5, dans laquelle l'agent pharmacologique est une hormone, de préférence un glucocorticoïde, minéralocorticoïde ou l'estrogène.

15. Composition selon l'une quelconque des revendications 1 à 14, comprenant en outre (d) un milieu aqueux pharmaceutiquement acceptable.

16. Composition pharmacologique selon la revendication 1, comprenant de l'éthanol absolu, un lipide et un agent anti-inflammatoire non stéroïdien, de préférence dans laquelle l'agent anti-inflammatoire non stéroïdien est l'indométhacine et de préférence dans laquelle l'indométhacine est présente à raison de 0,5 à 30 % en poids ou de 10 à 20 % en poids.

17. Procédé de préparation d'une suspension à partir de la composition selon les revendications 1 à 15, comprenant l'addition de ladite composition à un milieu aqueux pharmaceutiquement acceptable, de préférence en un rapport d'au moins 0,1:1 v/v, et de préférence dans lequel le milieu aqueux est l'eau, une solution de D-glucose à 5 % dans de l'eau, une solution de chlorure de sodium à 0,9 %, du tampon phosphate physiologique ou du tampon citrate physiologique.

18. Procédé selon la revendication 16, dans lequel ladite suspension contient des agrégats dans lesquels la concentration de l'agent pharmacologique, par rapport au lipide, est d'au moins 20 % en moles.

19. Procédé de préparation d'une composition en solution de lipide-agent pharmacologique selon l'une quelconque des revendications 1 à 15, comprenant
    (a) un lipide chargé privé de sels,
    (b) un solvant pour lipides, non aqueux, miscible à l'eau,
    (c) un agent pharmacologique lipososuble,
    le procédé comprenant l'étape de mélangeage de (a), (b) et (c).

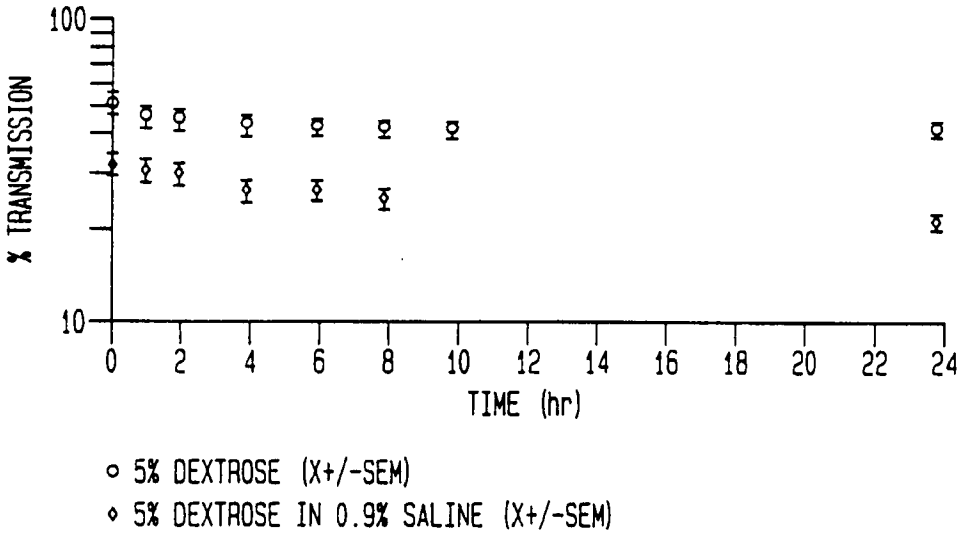
20. Procédé selon la revendication 19, comprenant en outre l'incorporation d'au moins 70 % en poids de phosphatidylcholine.

21. Procédé selon la revendication 19 ou 20, comprenant en outre l'addition de (d) un milieu aqueux pharmaceutiquement acceptable.

22. Procédé d'association, dans une composition pharmacologique, d'éthanol absolu, d'un lipide et d'un agent anti-inflammatoire non stéroïdien, selon la revendication 19, dans lequel l'agent anti-inflammatoire est l'indométhacine et de préférence dans lequel l'indométhacine est présente à raison de 0,5 à

environ 30 % en poids ou de 10 à 20 % en poids.

FIG.


100
10
% TRANSMISSION
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (hr)
○ 5% DEXTROSE (X+/-SEM)
◇ 5% DEXTROSE IN 0.9% SALINE (X+/-SEM)

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(54) **Noninvasive vaccination through the skin**

Nichtinvasive Impfung durch die Haut

Vaccination non invasive à travers la peau

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WO-A-91/01146 **WO-A-92/04009**

- **PAUL A, CEVC G: "Non-invasive administration of protein antigens: transdermal immunization with bovine serum albumin in transfersomes" VACCINE RESEARCH, vol. 4, no. 3, 1995, pages 145-164, XP002107365**
- **PAUL A ET AL: "Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes" VACCINE, vol. 16, no. 2-3, 2 January 1998, page 188-195 XP004098622**
- **CEVC G: "Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery" CRITICAL REVIEWS IN THERAPEUTIC DRUG CARRIER SYSTEMS, vol. 13, no. 3-4, 1996, pages 257-388, XP002107366**

EP 1 031 346 B1

Description

[0001] The present invention relates to novel vaccines for the non-invasive, transcutaneous administration of antigens associated with ultradeformable carriers, for the purpose of prophylactic or therapeutic vaccination. The vaccines comprise (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself, and (c) an antigen or an allergen. The invention further relates to methods for corresponding therapeutic or prophylactic vaccination of mammals.

[0002] Skin is the best accessible, but also the most difficult, entry into the body, due to the presence of the stratum corneum. This horny layer of the skin is an evolutionary optimised barrier which resembles the blood vessel wall, in that it comprises flaccid, tightly packed and laterally overlapping cells, whereby the basic cellular-tile motif in the stratum corneum is repeated 20-30 times. The intercellular contacts in the skin, moreover, are sealed with the densely packed and well organised blend of lipids. The stratum corneum therefore not only protects the organism from infections but also precludes an efficient uptake of antigens through the skin. This fact, which is advantageous from the point of allergy, prevented successful immunisation or vaccination through the intact skin to date.

[0003] The largest drugs on the market in any transdermal delivery device are smaller than 350 Da (Cevc, G. Drug delivery across the skin, Exp. Opin. Invest. Drugs (1997) 6: 1887-1937), as only such molecules can cross the tiny, self-sealing pores in the skin. The latter normally are less than 1 nm wide, when hydrophilic, or narrower, when hydrophobic. Organisms such as helminths therefore gain access into the body by penetrating the skin by using their biochemical machinery for the purpose of 'drilling holes' through the organ. Naturally occurring micro-lesions and shunts (such as pilosebaceous units) are available in the skin as well. However, they only cover up 0.1 % to 0.5 % of the skin surface and, consequently, do not contribute much to transcutaneous transport the fact notwithstanding that bacteria typically exploit such a route for a topical infection (Strange, P., Skov, L, Lisby, S., Nielsen, P. L., Baadsgard, O. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatoma. Arch. Dermatol. (1996) 132: 27-33.)

[0004] Only a few haptens exposed on the skin elicit a cutaneous immune response. This confirms that only sufficiently small molecules from a large load of the topically deposited haptens can find their way into the skin in an appreciable quantity. Such haptens then first irritate the organ and finally may cause hypersensitivity and contact dermatitis (Kondo, S., Sauder, D.N. Epidermal cytokines in allergic contact dermatitis. J. Am. Acad. Dermatol. (1995) 33: 786-800; Nasir, A., Gaspari, A. A. Contact dermatitis. Clinical perspectives and basic mechanisms. Clin. Rev. Allergy and Immunol. (1996) 14: 151-184). The problem is most serious with the low molecular weight chemicals or with the pharmaceuticals combined with skin irritants, such as skin permeation enhancers (Cevc,1997, *op. cit.*). Large molecules seldom are allergenic on the skin, owing to their limited ability to cross the barrier. A Th2 response to a highly immunogenic ovalbumin (Wang, L.-F., Lin, J.-Y., Hsieh, K.-H., Lin, R.-H. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with IgE production in mice. J. Immunol. (1996) 156: 4079-4082.) or to Cholera toxin (Glenn, G. M., Rao, M. Matyas, (1998) 391: 851; Glenn, G.M., Scharton-Karsten T, Vasell R, Mallet C.P., Hale T.L. and Alving C.R. Transcutaneous Immunization with Cholera toxin Protects Mice Against Lethal Mucosal Toxin Challenge . J. Immunol (1998) 161: 3211-3214.) was possible only after an epicutaneous exposure to a large amount of such proteins and was fairly weak. Moreover, the stratum corneum elimination from the skin was a prerequisite for producing detectable quantities of the specific antibodies against adenoviruses encoding the human carcinoembryonic antigen or human GM-CSF gene in 96 % or 43 %, respectively, of epicutaneously treated C57BL/6 mice (Deng, H., Qun, L., Khavari, P. A. Sustainable cutaneous gene delivery. Nature Biotechnology (1997) 15: 1388-1390.).

[0005] No protection against the above mentioned or other epicutaneously employed antigens was reported to date. Antibodies against diptheria or tetanus toxoid, and bovine serum albumin, which were generated by applying the antigens on the skin of BALB/c mice in combination with cholera toxin (Glenn et al., 1998, *op. cit.*) resulted in a very weak immune response without the adjuvant. Even after the inclusion of Cholera toxin (CT), the average specific antibody titre for diptheria and tetanus antigens was around 50x and between 70x and 4000x (depending on the inclusion of

individual data points), respectively, below that elicited by cholera toxin per se (Glenn et al., 1998, *op. cit*). The corresponding absolute respective titre values were 14±17 and 8±16; the anti-BSA titre was approximately 11±11 (average value +/- standard deviation as calculated from the published figures). No therapeutic or prophylactic effect was demonstrated for these low titres, which shows that the path towards simple non-invasive vaccination is not at all straightforward. The more recent paper published by the same group (Glenn et al., 1998b) demonstrated protection against CT after transnasal challenge which does not allow any conclusion with regard to protection obtainable by transdermal vaccination.

[0006] Earlier publications report on the deliverance of proteins across the skin, several orders of magnitude more efficient than in the above mentioned study, as judged by the titres, exploiting mechanosensitive and hydrosensitive, self-regulating carriers (Transfersomes) (for a review, see Cevc, 1997, *op. cit.*). For potent antigens this induced antibody titres that were comparable with those elicited by subcutaneous protein injections: in the case of BSA, the absolute titre of IgG was around 200 in either case (Paul, A., Cevc, G. Non-invasive administration of protein antigens. Epicutaneous immunisation with the bovine serum albumin. Vaccine Res. (1995) 4: 145-164) and for gap junction protein titres between 15.000 and 100.000 were measured (Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunisation with large proteins by means of ultradeformable drug carriers. Eur. J. Immunol. (1995) 25: 3521-3524; Paul, A., Cevc, G., Bachhawat, B. K. Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, Transfersomes. Vaccine (1997) 16: 188-195.). The review "Transferosomes, liposomes and other lipid suspensions on the skin" (Critical Rev. Therap. Drug Carrier 13, 257-388, 1996), inter alia, discusses DRV liposomes, as discribed in WO 92/04009, as immunoadjuvants in their own right. However, it is also disclosed that liposome-mediated penetration of cytokine IFN-gamma into the skin is inefficient and probably restricted to hair follicles. WO 91/01146 relates to immunogenic conjugates comprising a carbohydrate-containing antigen or other antigen bound to or genetically fused with a cytokine, lymphokine, hormone or growth factor. Yet, the generation of a protective immune response was not demonstrated in either of these publications and none of these publications provide for means and methods for the elucidation of a therapeutic or prophylactic immune response.

[0007] As is known today, the activity of Th1 or Th2 cells plays an important role in immune response: Th1 cells promote mainly the cell-mediated immunity, phagocyte-mediated host defence, but also the production of antigen specific IgG2a in mice. In contrast, Th2 cells tend preferentially to support phagocyte independent host-response, IgG1, IgE and IgA immunoglobulin generation.

[0008] The Th1 or Th2 basis of an immune response, that is, the differentiation into Th cell subtypes, not only depends on cytokines and the activity of other regulatory molecules (Luger, T. A., Schwarz, T. The role of cytokines and neuroendocrine hormones in cutaneous immunity and inflammation. Allergy (1995) 50: 292-302; Lohoff, M., Gessner, M., Bogdan, C., Roellinghoff, M. The Th1/Th2 paradigm and experimental murine *Leishmaniasis*. Int. Arch Allergy Immunol. (1998) 115: 191-202.); the nature of antigen presenting cells and antigen amount used also play an important role. Cytokines are produced transiently by almost all eukaryotic cells and act via specific cell-surface receptors. Indeed, every cell in the skin, after appropriate stimulation, can release such (glyco)protein factors or express their receptors. Most cytokines are pluripotent and can induce each other or else influence the expression of relevant receptors. This allows cytokines to act in synergistic, additive or antagonistic fashion, within the framework of so-called cytokine cascade (Luger & Schwarz, 1995; *op. cit.*).

[0009] The role of different cells in immunoactivation after cutaneous antigen application is as yet incompletely understood (Luger & Schwarz, 1995; *op. cit.;* Lohoff et al., 1998, *op. cit.*). Langerhans cells, located in the suprabasilar skin region, are believed to play the main role in immunopresentation. These cells first bind and process the antigens, then migrate from the epidermis into the lymphatic vessels, and further into the proximal, draining lymph node, bearing the digested antigens with them. During the process Langerhans cells undergo phenotypical and functional alterations and differentiate into (lymphoid) dendritic cells which finally offer the antigens to naive $CD4^+$ T cells that have entered the lymph nodes through the high endothelial venules. In contrast, the other two major types of antigen presenting cells in the skin, macrophages and B lymphocytes, first require activation in order to present antigens and stimulate T cells. Antibodies may be presented to T cells by the venular endothelial cells, and perhaps by certain basic cells of the skin as well.

[0010] It is clear, for example, that keratinocytes can augment the local inflammation by producing a plethora of proinflammatory cytokines, including IL-1α, GM-CSF and TNFα (Pastore, S., Fanales-Belaso, E., Abbanesi, C., Chinni, L.M., Giannetti, A., Girolomoni, G. Granulocyte macrophage colony stimulating factor is overproduced by keratinocytes in atopic dermatitis: Implications for sustained dendritic cell activation in the skin. J. Clin. Invest. (1997) 99: 3009-3017.). Keratinocyte derived cytokines are also critical for the maturation of Langerhans cells into potent antigen presenting cells (Nasir & Gaspari, 1996, *op. cit.*). The extent to which the former cells directly participate in antigen presentation (Kondo & Sauder, 1995, *op. cit.*) is unknown but the production of inhibitory cytokines, such as IL-10, non-functional IL-12 and TGFβ, by keratinocytes is an established fact (Nasir & Gaspari, 1996, *op. cit.*).

[0011] The fibroblast pool in the skin also contains cellular subsets that are involved in antigen processing. For example, one subset of fibroblasts is recruited selectively by cytokines at the inflammation site in scleroderma (Fries,

K.M., Blieden, T., Looney, R. J., Sempowski, G.D., Silvera, M.R., Willis, R. A., Phipps, R. P. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. Clin. Immunol. Immunopathol. (1994) 72: 283-292.).

[0012] It has been reported previously that epicutaneous antigen application produces a different immune response than the more conventional routes of administration through the oral cavity or the nose. For example, after repeated epicutaneous ovalbumin exposure on the skin anti-ovalbumin IgE-s are prominent (Wang et al., 1996, *op. cit.*). Using bovine serum albumin as a model antigen on the skin, an unusually strong IgA production was previously observed (Paul et al., 1997, *op. cit.*), but no consistent picture of the interdependency between the details of epicutaneous antigen presentation and the resulting immune response emerged to date.

[0013] Numerous and different cells participate in mounting an immune response against the cutaneously delivered macromolecules. As has been stated above, the approaches taken so far have not led to the establishment of a convincing strategy for generating a protective immune response. This may be due to the fact that the prior art strategies, such as antigen injection, have not assisted in dissecting the immune response obtainable by applying antigens to the skin to an extent that allows for devising a directed and protective immune response. For example, it is known that antigen injection, as any lesion or other kind of skin perturbation, including the presence of chemical irritants, releases various cytokines from the skin (which not only is the heaviest organ in the body but also makes out the major part of the body immune system). This maximises the strength, but prevents the fine tuning, of cutaneous immune response, which is also sensitive to the nature of antigens used. High impact vaccine delivery profits from this effect.

[0014] Material transport across the skin by means of ultradeformable carriers is just the opposite of said high-impact delivery approach, as it reportedly does not affect the skin. It is believed that this is clue to the fact that such hydrosensitive, ultradeformable bodies - so called Transfersomes™ (Cevc, 1997, *op. cit.*), penetrate the stratum corneum through 'virtual channels' between corneocytes, adjusted to the shape of the cells (Schätzlein, A., Cevc, G. Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes). Br. J. Dermatol. (1998) 138: 583-592.). It was proposed that Transfersomes push the cells in the skin and intercellular lipids apart during the process, preferentially at the sites of weakest contact. The passages thus generated seem to be approximately 20-30 nm wide, on the average. They cover several percent (~4%) of the skin surface (Schätzlein & Cevc, 1998, *op. cit.*), the draining of adjacent surface not included. This is much more than the normal shunt area (~0.1%), which explains the quantitative differences between the anti-BSA titres measured after antigen administration with ultradeformable carriers (Paul & Cevc, 1995, *op. cit.*) or by using Cholera toxin as an adjuvant (Glenn et al., 1998a, b, *op. cit.*).

[0015] Virtual channels in the skin opened by the carriers appear to be sufficiently wide to let the carriers as well as material associated with them pass through the barrier without significantly perturbing the organ. However, repeated insulin delivery across the skin by means of ultradeformable carriers was found not to induce antibodies against the protein (Cevc, G., Gebauer, D., Schätzlein, A. Blume, G. Ultraflexible Vesicles, Transfersomes, Have an Extremely Low Permeation Resistance and Transport Therapeutic Amounts of Insulin Across the Intact Mammalian Skin. Biochim. Biophys. Acta (1998) 1368: 201-215.)

[0016] The technical problem underlying the present invention was therefore to establish a means that allows for the successful induction of a medically useful transdermal immune response. The solution to said technical problem is achieved by providing the embodiments characterised in the claims.

[0017] Accordingly, the present invention relates to a transdermal vaccine comprising (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a, preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, (b) a compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity or exerts such an activity, either of which resulting in the desired, medically useful immune response, and (c) an antigen or an allergen.

[0018] As regards the above recited values of up to 99%, it is to be noted that values below 50 % of the former relative concentration are often used. Even more advantageously values below 40 rel-% or even around and below 30 rel-% are chosen, whereas with the droplets that cannot be solubilised by the more soluble component relative concentrations that exceed the above mentioned ones by the factor of up to 2 are preferred.

[0019] In the context of this invention, the term "pathogen" refers to an entity which through its presence in or on the

body leads to or promotes a pathological state which, in principle, is amenable to or could profit from a preventive, curative or adjuvant immunotherapy. This includes pathogens causing microbial diseases such as extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species; a number of bacteria and all viruses, which survive and replicate within host cells; this latter group encompasses mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including but not limited to hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox, (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and various fungi prospering inside host cells; parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites. The pathogens further include *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases. Pathogens in this invention, furthermore, are assumed to include, but are not limited to, the eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body which do not result from microbial infections. Parts of certain pathogens, especially various microbial toxins, have porin-like properties and, consequently, may have some capability to cross the mucosa or to increase the flexibility of penetrant membranes.

[0020] The term "specifically" in combination with "releases" or "induces" denotes the fact that the compound interacts with cells capable of releasing cytokines by a receptor-mediated triggering of this cytokine release or induction. This specific release or induction is in contrast to an unspecific release or induction that is, for example, obtained by an intradermal injection.

[0021] The term "allergen" is used in this invention to describe materials of endogenous or xenogenic, e.g. animal or plant, origin which result in an undesired immune response of the body exposed to such an allergen, often resulting in an acute hypersensitivity reaction. Allergising microbes or parts thereof (e.g. of mite), parts of plants (e.g. pollen) or animal (e.g. hair and skin debris), but also man made and inorganic substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the skin or not.

[0022] The term "(therapeutic) vaccination" in the context of this invention describes any kind of therapeutic immunisation, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

[0023] Immunisation denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

[0024] An "antibody" or an "immunoglobulin" denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their "derivatives" include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives. Fragments include e.g. single chain fragments, Fc-, Fab- $F(ab')_2$- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

[0025] An "antigen" is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system.

[0026] The term "cytokine", as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1α and IL-1β, tumour necrosis factor (TNF), transforming growth factor (TGF-β and -α), Type I and II interferons (IFN-α1, IFN-α2, (IFN-ω), IFN-β, IFN-γ), migration inhibitory factor, MIF, c-kit ligand, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

[0027] Cytokines that mediate natural immunity particularly well include type I interferons (IFN-α and IFN-β), tumour necrosis factor (TNF), interleukin-1 (IL-1 α and IL-1β), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. The process relies on antiproliferative (e.g. with IFN-s), proinflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, conse-

quently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic action.

[0028] Cytokines that mediate immune-mediated inflammation, which heavily relies on the cell-mediated response, are interferon-gamma (IFN-γ), lymphotoxin (TNF-β, interleukin-10 (IL-10), interleukin-5 (IL-5), interleukin-12 (IL-12) and, probably, migration inhibition factor. Leukocyte growth and differentiation are most affected by interleukin-3 (IL-3), c-kit ligand, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage or granulocyte colony stimulating factor (M-CSF or G-CSF) and interleukin-7 (IL-7).

[0029] The term "immunoadjuvant" is used here to describe any substance which supports, augments, stimulates, activates, potentiates or modulates the desired immune response of either cellular or humoral type, specifically in the case of prophylactic treatment by increasing the antigen specific immune response of any kind and in the case of therapeutic treatment often by supporting cell-mediated immunity. This can be achieved by the addition of suitable cytokines, their blends or antagonists, or less directly by the chemical irritation of the skin, when this contributes directly or indirectly to the release of cytokines from the skin or other involved peripheral tissues, or else by catalysing or promoting the biosynthesis of the molecules in the tissue which then lead to such action, provided that the final outcome is an increased success of vaccination, that is of prophylactic and/or therapeutic action of used antigen. The class of immunoadjuvants which indirectly contribute to the useful cytokine pool includes small chemical entities with an allergenic potential, such as certain allergenic (metal) ions, including but not limited to LiCl, $HgCl_2$, molibdenum, acids, bases and other irritating compounds, such as dicyclohexylmethane-4,4'-diisocyanate, ditrocarb (diethyldithiocarbamate), 2,4-dinitrochlorobenzene, isoprinosine, isophorone-diisocyanate, levamisole, (phenyl)oxazolone and alike, Swansonine, sizofran, phthalic anhydride, thymopentin, (fatty) alcohols, (fatty) amines, (fatty) ethers, ricin, or other suitable amphiphiles, many surfactants and chemical skin permeation enhancers, as well as derivatives or combinations thereof; furthermore, (low molecular weight) fragments of or derivatives from microbes, including lipopolysaccharides (such as LPS), cord-factor (trehalose-dimycolate) and other polysaccharides attached to membranes, when used in sufficient quantity;, acetylmuramyl-alanyl-isoglutamin, and arger fragments of microbes, including bacterial exo- and endotoxins, or enterotoxins, such as cholera toxin and the heat labile toxin of *E. coli,* and their macromolecular fragments, such as A-chain derivatives, most, if not all, of which seem to posses ADP-ribosylating activity, the high potency immunoadjuvant LT holotoxin, etc., cell-wall skeleton, attenuated bacteria, such as BCG, etc. Less established examples include clostridial toxin, purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* outer membrane protein of group B *Neisseria meningitidis* (GBOMP), various other peptidoglycanes, etc. Immunoadjuvants, in other words, include molecules that alter the uptake or presentation of antigens, activate or increase the proliferation of antigen specific lymphocytes, or interfere with the dominant control mechanism in the immune response, not just in the skin but also in the other immunocompetent tissues. (The mucosal adjuvant activity of ADP-ribosylating bacterial enterotoxins is a well established and known example for this.) On the other hand, molecules which change the (relative) concentrations of cytokines or other immunoadjuvants, such as anti-immunoadjuvant antibodies or other agonists or antagonists of immunoadjuvants, also are immunoadjuvants in the sense of this invention. The same is true for molecules which affect lymphocyte homing, such as various selectins (LECAMS, e.g. various CD62-s), GlyCAM-1, MadCAM-1, VCAM-1, ICAM-1, hyaluronate, etc., and other chemokines, such as RANTES or MCP-1. Endogenous group of immunoadjuvant furthermore comprises histamines, transfer factor, tuftsin, etc.. As many of the above mentioned immunoadjuvants do not have sufficient potency to ensure the desired effect after the non-invasive immunisation at too low, and sometimes too high, concentration or on their own, the functional definition of an aduvant used in this work includes a fortiory sufficient and such modulation of cytokine concentration and distribution pattern in the body that results in mounting the desired therapeutic or prophylactic immune response. If required to gain clarity said modulation and its extent must be determined in a dedicated experiment, in which the specific cytokine levels are determined, for example.

[0030] "Immunoadjuvant manipulation" denotes a non-chemical treatment of the skin, such as skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound, field, etc., or even an injection of a non-immunogenic formulation in the skin, provided that such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

[0031] The term "immunogen" denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing an immune response.

[0032] "Immuno-tolerance" denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

[0033] Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

[0034] Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

[0035] The term "two forms of a substance" in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc..

[0036] "Non-invasive administration" or "non-invasive delivery" denotes application on or transport through an intact

barrier, in the biological applications dealt with in this disclosure, through intact skin.

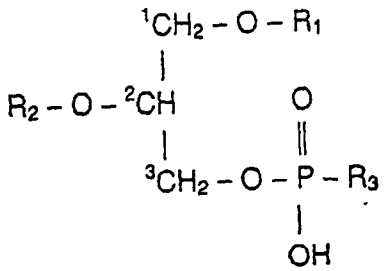
[0037] "Penetration" describes a non-diffusive motion of relatively large entities across a barrier. This process typically relies on the penetrant adaptation to the otherwise confining pores in the barrier and also may involve a penetration induced decrease in the barrier resistance, such as pore widening or channel opening; the process does not depend, however, primarily on the penetrant concentration gradient across the barrier.

"Permeation" refers to a diffusive motion across the semi-permeable barriers. The prime example for this is the transport of molecules or molecular aggregates under the influence of a permeating species concentration gradient across the barrier.

[0038] A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

[0039] For further definitions, especially such pertaining to the penetrants in terms of complex body deformability, the corresponding mechanism of action, lists of interesting penetrant ingredients or selected agents it is referred to the issued or pending patents (DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287). Detailed information relevant for the manufacturing process and penetrant loading with the antigenic (macro)molecules and/or immunoadjuvants, which are too big to permeate through the barrier, can be found in international patent application PCT/EP98/06750.

[0040] Typically, the less soluble amongst the aggregating substances forming a carrier is a lipid or lipid-like material, especially a polar lipid, whereas the substance which is more soluble in the suspending liquid and which increases the droplet adaptability belongs to surfactants or else has surfactant-like properties. The former ingredient, typically, is a lipid or lipid-like material from a biological source or a corresponding synthetic lipid or any of its modifications, such lipid often belonging to the class of pure phospholipids with the chemical formula



where R_1 and R_2 is an aliphatic chain, typically a C_{10-20}-acyl, or -alkyl or partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoyl chain, and where R_3 is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl, C_{1-4}-alkyl, C_{1-5}-alkyl substituted with carboxy, C_{2-5}-alkyl substituted with hydroxy, C_{2-5}-alkyl substituted with carboxy and hydroxy, or C_{2-5} -alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid comprising also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or carbohydrate-containing lipids, or any other bilayer forming lipids, in particular half-protonated fluid fatty acids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingomyelins or other sphingophospholipids, glycosphingolipids (including cerebrosides, ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides or other glycolipids or synthetic lipids, in particular with or else with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyllipids.

[0041] The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyldimethyl-aminoxida, esp. a dodecyl-

dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N- dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitan-monooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl, -myristoyl, -cetylstearyl, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate, -linoleate, -linolenate-, - palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkyl-sulphate (salt), e.g. in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g. lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecyl-phosphatidylcholine, or else is a surface-active polypeptide. It is important to realise, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionisation or salt states of polar lipids differ widely in their properties. It therefore stands to reason that two different physicochemical states of the same (polar) lipid mixed together in a membrane will produce a highly deformable carrier satisfying the conditions of this work.

[0042] More general information on lipid suspensions can be found in handbook dealing with 'Liposomes' (Gregoriadis, G., Hrsg., CRC Press, Boca Raton, Fl., Vols 1-3, 1987), in the book Liposomes as drug carriers' (Gregoriadis, G., Hrsg., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids which can be used conveniently to prepare biocompatible immunopenetrants are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

[0043] It may be convenient to adjust the pH value of a formulation immediately after preparation or just before its application. Such an adjustment should prevent the deterioration of individual system components and/or drug carriers under the conditions of initial pH without sacrificing physiological compatibility. To neutralise a penetrant suspensions it is reasonable to use biocompatible acids or bases to prepare buffers with a pH value between 3 and 12, frequently between 5 and 9 and most often between 6 and 8 Physiologically acceptable acids are, for example, diluted aqueous solutions of mineral acids, such as hydrochloric acid, sulphuric acid, or phosphoric acid, or organic acids, such as carboxyalkane acids, e.g. acetic acid. Physiologically acceptable bases are, for example, diluted sodium hydroxide, suitably ionised phosphoric acid, etc.

[0044] If required, immunogen suspension can be diluted or concentrated (e.g. by ultracentrifugation or ultra-filtration) before the application; additives can also be given into the suspension at this time or before. The additives are often selected amongst substances that reduce the formulation sensitivity to ambient stress, including microbicides, antioxidants, antagonists of undesired enzyme action, in case cryo-preservants, thickening agents, etc.. However, after any system manipulation, the carrier characteristics should be checked and, if required, readjusted.

[0045] In accordance with the present invention it was surprisingly found that macromolecular antigens associated with ultradeformable lipid aggregates described herein (immuno-penetrants) can cross artificial porous barriers as well as the skin, despite the fact that the average diameter of said penetrants exceeds the average pore/channel diameter, and that such immuno-penetrants can elicit a therapeutic or prophylactic immune response, provided that said immuno-penetrants are associated with compounds that display cytokine activity or induce the generation in and/or the release of cytokines from the skin, and/or other immunocompetent organs in the body. Alternatively, said compounds antagonize cytokine activity. This latter embodiment advantageously directs the immune response into a Th1 or Th2 dependent immune response by blocking the respective other route. The antigen carriers described in this invention maintain sufficient stability before and during the process. It was further surprisingly found that the resulting immune response is not directly proportional to the applied dose, which implies that the amount of antigen may be varied. Said amount should be well chosen for optimum effect. Choosing optimum amount or range of antigen is well within the skills of the person knowledgeable in the art taking into account the teachings of this specification.

[0046] The at least bi-component immuno-aggregates used as carriers in the vaccines of this invention excel in high deformability and most often have the form of vesicles a with highly flexible membrane. Although such carriers have been employed in immunisation protocols before, it was unexpected that the previously postulated carrier immunoadjuvancy does not eliminate the need to include compounds with cytokine activity or the appropriate antagonists thereto, preferably of IL-4, IL-10, TGF-β, IL-5, IL-6, IL-9 and IL-13; or, after the pre-stimulation of T-cell receptor, also of IL-1 in order, to achieve the desired protective immune response. By the same argument, IL-12, IFN-γ and lymphotoxin (LT or TNF-β) are advantageously included to promote the Th1 response and thus to favour the cell-mediated immune

response and to provide a means for treating viral and other parasite diseases or for promoting the immuno-tolerance. For example, a combination of IFN_γ, IL-12 and anti IL-4 is expected to revert Th2 response toward Th1 type. More broadly speaking, increasing the relative amount of IL-12 and IL-4 in the beginning of an immune response in favour of the former is proposed to be useful to promote Th1 response also in the case of penetrant mediated immunisation, and *vice versa*, whereas IL-2 is going to support NK and B cells growth, to stimulate antibody synthesis, and to affect the magnitude of T-cell dependent immune response in general. Thus, whereas the prior art demonstrated that antibody titres could be induced by using suitable carriers in combination with antigen and optionally with immunoaduvants, the immuno responses obtained were not demonstrated to be protective.

[0047] A particular advantage of the present invention is due to the fact that it was surprisingly found that the transfer of the penetrant described in this invention does not lead to an essential disturbance of the cytokine composition within the skin. In other words, the transfer of these carriers through the skin will per se not induce any essential release of cytokines. It is therefore possible to study now and to trigger a desired immuno response by including into the vaccine of the invention a compound that specifically induces or releases cytokines from cells in the skin or other organs that are competent to release such cytokines. Fine tuning of a desired immuno response thus may be possible. Alternatively, a compound having or exerting cytokine activity can be included into the vaccine of the invention. Further, an antagonist of cytokine activity may be used that specifically prevents the action of such cytokines. In this embodiment, the immuno response may advantageously be directed towards the Th1 or Th2 pathway. It is important to note that these compounds specifically induce or release cytokines in dependence on antigen properties. They are thus distinguished from adjuvants which, in accordance with the present invention, unspecifically and broadly support an immuno response.

[0048] Application of the vaccine of the present invention allows, in conclusion, therefore the fine tuning of a desired immuno response to a given antigen, the nature of which also plays a given role, of course. This immuno response may be enhanced by an unspecific immuno response, triggered, for example, by an adjuvant. The option of fine tuning the immuno response is in particular advantageous over the prior art using only the injection of vaccines because the injection process per se will heavily and unspecifically disturb the relative cytokine concentrations in the skin.

[0049] Consideration of the above mentioned criteria not only provides the basis for a suitable kind of immunopresentation to the cells of the skin and peripheral immune system (by the carriers) but also ensures such immunoprocessing that will either predominantly generate antigen-neutralising antibodies in the body, will give rise to the cell-mediated immune response, or else will result in the gradual development of tolerance against the antigens or in the specific promotion of cell-mediated immunity.

[0050] In accordance with the present invention, it was also found that the outcome of noninvasive transcutaneous vaccination is strongly affected by the immunopenetrant (antigen carrier) composition. Using antigens of different purity, unexpectedly, resulted in vastly different immune response. This was reflected in the observation that organisms with a similar overall titre revealed diverse levels of protection, probably due to the different final antigen isotype patterns.

[0051] Furthermore, the addition of a conventional, low molecular weight immunoadjuvant, monophosphoryl lipid A, not only made the result of epicutaneous immunisation more robust, as documented by the smaller standard deviation in the measured antibody titres published before. Using this immunoadjuvant in immuno-carriers also, unexpectedly and contrary to previous experience obtained in mice, increased the secretion of IgG2b, and less strongly of IgG2a, but did not enhance IgA production. As the presence of IgG1, which is a Th1-like immunoglobulin, is inferred to be essential for, at least murine, protection against the tetanus toxin, the role of lipid A or bacterial antigens was thus revealed for the first time. For the future medical and commercial use of teachings disclosed in this invention it is important to realise that a high (specific) antibody titre does not necessarily imply a good protection result; to achieve the desired and sufficient protection the right kind and relative amount of certain antibody isotypes is required, such that will give prevailantly Th1- or Th2-type of immune response (see previous discussion), as the case should be.

[0052] Basic formulations suitable for achieving the desired goals are known in the art; see, e.g., DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, DE 44 47 287, for more detailed or complementary information. The vaccine of this invention is not useful just for prophylactic or therapeutic vaccination but, moreover, is applicable for the treatment of allergy and for obtaining immunity against microbes, including extracellular and intercellular bacteria, viruses and parasites in the human and veterinary medicine.

[0053] In combination with the above mentioned penetrants, an antigen, such as an immunoactive substance, is transported across the barrier in form of a physical or a chemical complex with the former.

[0054] In order to profit from the pool of cytokines residing in the skin, a particularly useful method of vaccination is proposed in which an immunogen is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation as defined before.

[0055] It is particularly advantageous to use the readings from the above mentioned local immune response to a patch assessment for optimising the details and the course of further allergen administration, and thus to positively affect the outcome of therapeutic or prophylactic vaccination. It is believed that such an approach could be used advantageously to reach or improve immuno-tolerance of the tested subject to an applied allergen.

[0056] If primary immunisation is done invasively, typically by using a subcutaneous injection or some other suitable

skin barrier perforating/destructing method, , one expects to obtain high IgM levels but the subsequent, booster immunisations may then be done non-invasively as described in this invention.

[0057] Finally, several optimisation methods are proposed which can be used to improve immunogens and vaccination based on highly deformable penetrants. Preferred is a method wherein the flux of penetrants associated with an immunogen through the various pores in a well-defined barrier is determined as a function of suitable driving force or pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further. Its core is the determination of the flux of immunopenetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which acts across the barrier, and the resulting data analysis in terms of a characteristic curve which, in turn, can be employed to optimise the formulation or application further, based on comparison of different data sets. This includes comparison with the results pertaining to the immunogen-free penetrant suspensions of known skin penetration capability, reported for example by Cevc et al., (1998, *op. cit.*). In a complementary, preferred, embodiment various combinations of immunomodulants or of immunomodulating procedures are tested with regard to chiefly Th1- or Th2-related cytokine production and the results are then used to make a suitable choice for the final therapeutic or prophylactic application.

[0058] Vaccination is typically done at ambient temperature, but lower or higher temperatures may also be suitable. They make particular sense with the formulations comprising synthetic substances which are rigid between the room and the skin or other barrier temperature.

[0059] Manufacturing temperature is normally chosen in the 0 to 95 °C range. Preferably, one works in the temperature range 10-70 °C, most frequently at temperatures between 15 °C and 45 °C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in composition or physical state. The skin temperature is normally 32 °C. Other temperature ranges are possible, however, most notably for the systems containing freezable or non-volatile components, cryo-or heat-stabilised formulations, etc.

[0060] If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in cold (e.g. at 4°C), with or without an associated antigen. Manufacturing and storage under an inert atmosphere, e.g. under nitrogen, is possible and sometimes sensible. The shelf-life of immunogen formulation can also be extended by using substances with only a small number of double bonds, that is, by a low degree of unsaturation, by the addition of antioxidants, chelators, and other stabilising agents, or by preparing the immunopenetrants ad hoc or *in situ* from a freeze dried or a dry mixture.

[0061] In a preferred embodiment of the vaccine according to the invention the compound which specifically releases or specifically induces molecules with cytokine or anti-cytokine activity and the antigen are associated with the penetrant.

[0062] In a further preferred embodiment of the vaccine according to the present invention the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

[0063] In an additional preferred embodiment of the vaccine according to the present invention the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.

[0064] The invention in one further preferred embodiment relates to a vaccine wherein total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 w-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.

[0065] In another preferred embodiment of the vaccine according to the present invention total antigen concentration is between 0.001 w-% and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.

[0066] In another preferred embodiment of the vaccine according to the present invention the formulation further comprises (da) a low molecular weight chemical irritant, and/or (db) a low molecular weight compound from a pathogen or a fragment or a derivative thereof.

[0067] In yet another preferred embodiment of the vaccine according to the present invention the compound exerting cytokine activity is IL-4, IL-2, TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, preferably IFN-α or IFN-β, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

[0068] In one more preferred embodiment of the vaccine according to the present invention the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.

[0069] The term "active fragment or derivative thereof" in this connection means that the above-recited activity is essentially maintained or mimicked by the substance used.

[0070] In another preferred embodiment of the vaccine according to the present invention the antigen is derived from a pathogen.

[0071] In another particularly preferred embodiment of the vaccine according to the present invention said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus*, gram-

negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.

[0072] In a preferred embodiment of the vaccine according to the present invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant which leads to an acute hypersensitivity reaction of the body exposed to the allergen, many such allergens stemming from mite, polen, animal hair or skin debris, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

[0073] In a further preferred embodiment of the vaccine according to the present invention the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen and immunoadjuvant chosen, performed by subcutaneously injecting the formulation or performing the tests *in vitro,* and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

[0074] In a different preferred embodiment of the vaccine according to the present invention the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo-and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli,* an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

[0075] In a particularly preferred embodiment of the present invention said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.

[0076] In another particularly preferred embodiment of the vaccine according to the present invention the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

[0077] In still another particularly preferred embodiment of the vaccine according to the present invention the low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines, (fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

[0078] In a preferred embodiment of the vaccine according to present invention the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or comparable subject is deemed to be unacceptable owing to the local irritancy, as assessed by the methods and standards commonly used to test such an irritant.

[0079] In a further particularly preferred embodiment of the vaccine according to the present invention the allergen belongs to the class of inhalation allergens, including various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; food and drug allergens; contact allergens; injection, invasion and depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., parts of implantation material, etc..

[0080] In a preferred embodiment of the vaccine according to the present invention the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunisation, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.

[0081] In another preferred embodiment of the vaccine according to the present invention the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2}, even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2}, and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2}. It may also be advantageous to use different administration areas to control the applied immunogen dose, using easily accessible or sheltered body areas (such as the chest or back regions, arms,

lateral side of the neck, e.g. behind the ears, or even in the scalp region) for the purpose.

[0082] In a different preferred embodiment of the vaccine according to the present invention said antigen is a pure or purified antigen. The use of highly purified antigens in the vaccine of the invention has turned out to be particularly advantageous for the generation of a protective imrnuno response.

[0083] The present invention further relates to a kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine.

[0084] In a preferred embodiment according to the present invention the kit comprises at least one injectable dose of the antigen described above.

[0085] The present invention further relates to a method for generating a protective immune response on a mammal comprising vaccinating said mammal with a vaccine as described above.

[0086] In another preferred embodiment of the method according to the present invention different treatment areas are selected to control the applied immunogen dose and the outcome of therapeutic vaccination.

[0087] In one more preferred embodiment of the method according to the present invention a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.

[0088] In a different preferred embodiment of the method according to the present invention the vaccine of the present invention is applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

[0089] In a preferred embodiment of the method according to the present invention immunogen is applied in a non-occlusive patch. This embodiment can also be used for the purpose of assessing the skin reaction to an epicutaneously administered immunogen in the penetrant suspension, to which the former, at least originally, is allergic and which thus gives rise to an acute local hypersensitivity reaction, as seen, for example from the resulting flare, irritation, etc.

[0090] In another preferred embodiment of the method according to the present invention at least one dose of vaccine is administered.

[0091] This embodiment of the method of the invention includes the repeated administration of the vaccine of the invention. Repeated administration includes repeated administration on the skin or one or more administrations on the skin in combination with e.g. parenteral administrations. In this connection, the kit of the invention may be advantageously used that comprises one or more containers or ampules comprising the vaccine of the invention.

[0092] In a particularly preferred embodiment of the method according to the present invention said vaccine is administered as a booster vaccination.

[0093] In a most preferred embodiment of the method according to the present invention the primary immunisation is done invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destructing method, and the at least one subsequent, booster immunisation is done non-invasively.

[0094] In a preferred embodiment of the method according to the present invention the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.

[0095] In a particularly preferred embodiment of the method according to the present invention the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years. In a further preferred embodiment, repeated immunogen administration is advocated to maximise the final effect of a therapeutic vaccination. It is proposed to use between 2 and 10, often between 2 and 7, more typically up to 5 and most preferred up to 3 immunisations, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined as described above or another suitable assessment method, or else to deem the effort as having failed. The time interval between subsequent vaccinations should preferably be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years, when a subject is being immunised for the first time. Rodents, such as mice and rabbits are advantageously immunised in 2 weeks interval, primates, e.g. monkeys and often humans, need a booster vaccination in 3-6 months interval.

[0096] In a preferred embodiment of the method according to the present invention the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

[0097] The invention finally relates to the use of the transdermal carrier, the compound which specifically releases

or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined hereinbefore for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

[0098] The disclosure contents of the documents cited throughout this specification are herewith incorporated by reference. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of Innovative Dermale Applikationen GmbH and bearing the title "Transnasal transport/immunisation with highly adaptable carriers".

[0099] The figures show:

[0100] Figure 1 gives the data on survival of animals immunised epicutaneously with mixed micelles or Transfersomes loaded with TT, to illustrate aggregate size (stability) effect, since the over-destabilised Transfersomes normally disintegrate into the mixed lipid micelles.

[0101] In figure 2 the comparison is made between the immune response to conventional lipid vesicles (liposomes) and ultradeformable lipid vesicles (Transfersomes) carrying TT and applied on the skin, the information on corresponding specific antibody concentrations in serum (expressed as absorbance) being given in upper panel.

[0102] Figure 3 illustrates the effect of increasing antigen dose on the outcome of epicutaneous immunisation by means of Transfersomes, the results being expressed as absorbance change, antibody titre, or animal survival, together with the corresponding specific antibody isotyping data.

[0103] Figure 4 highlights the effect of antigen purity on the result of epicutaneous immunisation with tetanus toxoid in Transfersomes, including information on time dependence of animal survival.

[0104] Figure 5 compares the outcome of repeated invasive (subcutaneous) and non-invasive (epicutaneous) immunisation by means of TT in Transfersomes, including animal survival, serum concentration (in terms of absorbance), specific antibody titre, and antibody distribution pattern values.

[0105] Figure 6 illustrates the effect of skin pre-treatment (non-specific challenge) on the immune response following Transfersome mediated TT delivery across the skin.

[0106] Figure 7 focuses on adjuvant effect of a relatively low-molecular weight immuno-stimulator, monophosphoryl Lipid A (LA), delivered across intact skin together with TT in Transfersomes.

[0107] Figure 8 demonstrates the immuno-adjuvancy of a cytokine, interleukin-12 (IL-12) transported across the skin together with TT by means of Transfersomes.

[0108] Figure 9 deals with the immuno-modulation by various cytokines of the murine response against TT antigen delivered in Transfersomes non-invasively through the skin.

[0109] Figure 10 presents experimental evidence for the immune response stimulation of mice treated on the skin by TT in Transfersomes, when the carriers also include cholera toxin (CT) to support the specific antibody production, and thus animal protection against an otherwise lethal challenge by the tetanus toxin.

[0110] The documents cited in this specification are incorporated herein by reference.

[0111] The examples illustrate but do not define the limits of the invention.

General experimental set-up and sample preparation

[0112] ***Mice*** *of Swiss albino* strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunisation and were normally kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. One day prior to an immunisation, the application area on murine back was shaved carefully. The antigen was administered with a high precision pipette on the skin surface and left to dry out partially. To prevent immunogen abrasion, the animals were transferred into individual cages in which they were kept for 18 hours following each epicutaneous material administration.

[0113] General anaesthesia was used to keep the test animals stress free and quiet during manipulations, including immunisation. An injection of a mixture of Ketavet and Rompun (0.3 mL per mouse of an isotonic NaCl solution containing 0.0071 % Rompun (Bayer, Leverkusen, Germany) and 14.3 mg/mL Ketavet (Parke-Davis, Rochester, N.Y) into the peritoneal cavity was used for the purpose. This typically kept the animals asleep for app. 2 hours.

Immunogens.

[0114] Ultradeformable immuno-carriers, or immuno-penetrants (immuno-Transfersomes), studied in this work, typically had the form of (oligo)bilayer vesicles. They contained biocompatible (phospho)lipids, such as phosphatidylcholine, and (bio)surfactants, such as sodium cholate or polysorbate (Tween 80), different compositions maintaining the high aggregate deformability being possible. Additional ingredients were monophosphoryl lipid A, with a versatile immunoadjuvant activity, and antigens, as required and specified.

[0115] *Conventional vesicles, liposomes,* comprised soy phosphatidylcholine (SPC; Nattermann Phospholipids,

Rhone-Poulenc Rorer, Cologne, Germany) and were prepared as described as follows. An organic lipid solution with or without the adjuvant monophosphoryl lipid A (MLA) at 0.04 mol-% relative to SPC was first dried under vacuum (10 Pa, overnight). The resulting lipid film was hydrated with a solution of tetanus toxoid (2.0 mg/mL; Accurate antibodies, NY, USA) in phosphate buffer (pH = 6.5) to get a 10 wt-% lipid suspension. Crude suspension of lipid vesicles was extruded through the series of polycarbonate membranes with 800 nm, 400 nm, and 200 nm pores, to narrow down the final vesicle size distribution.

[0116] *Highly deformable vesicles, Transfersomes,* were prepared as described earlier (Paul et al., 1995 *op. cit.*). In short, an ethanolic SPC solution was mixed with sodium cholate (Merck, Darmstadt, Germany) (3.75/1 mol/mol) and the adjuvant, if required. The mixture was dispersed in 10 mM phosphate buffer (pH = 6.5). This was done with tetanus toxoid present in the solution to give between 0.25 mg and 2.0 mg protein per 1 mL of suspension, as required. Vesicle suspension was then frozen and thawed three times. Subsequently, the formulation was passed through a microporous filter (200 nm; Poretics, CA) under pressure. To check the reproducibility of vesicle manufacturing, the optical density at 400 nm was measured with each preparation and confirmed to be approximately constant.

[0117] By varying surfactant-to-lipid ratio the vesicular aggregate deformability was controlled, up to the concentration at which membranes became unstable, owing to the high surfactant concentration, and reverted into a micellar form. Lipid vesicles without the surfactant added, which are commonly known as liposomes and have at least 10x less flexible membranes than Transfersomes, were used as negative controls.

[0118] Total lipid concentration was typically 10 w-%, unless stated otherwise. Antigen concentration was typically, but not necessarily, of the order of 1 mg/mL. A buffer containing microbicide provided the bulk phase. For other suitable compositions the expert is explicitly referred to other publications and patents from our laboratory.

[0119] ***Immunisations*** were done with different formulations, including the ultradeformable vesicles without antigens; such vesicles then contained the tetanus toxoid (with or without lipid A) and free immunogen. Each formulation was tested on six mice, unless stated otherwise.

[0120] In the case of subcutaneous immunisation, 40 μg of immunogen was injected per mouse. For a non-invasive administration, tetanus toxoid doses between 1 μg and 80 μg, associated with different carriers, were administered per mouse on the intact skin of upper dorsum. All non-injected formulations were applied with a high precision pipette and left to dry; during this period mice were kept in separate cages to minimise the applied material abrasion, such as might result from the rubbing of the murine backs on each other. Animals were boosted every two weeks, that is on days 14 and 28; the total immunisation scheme thus consisted of three doses, and comprised a prime and two boosts.

[0121] Animals were bled retro-orbitally on the days 7, 21 and 35. The collected blood was first allowed to clot. After a brief centrifugation in a micro-centrifuge the serum was separated, de-complemented at 56 °C for 30 min, and then stored at -20 °C, until the total antibody concentration and the specific antibody isotypes was determined.

[0122] ***Absorbency measurements*** were done using standard UV-vis spectrometer.

[0123] ***Measurement of tetanus toxoid (TT) specific antibodies in serum by ELISA.*** The level of anti-tetanus antibodies was determined by ELISA in the customary fashion, typically in a duplicate. In brief, ELISA plates (maxisorp: NUNC, Germany) were coated with an aliquot (100 μL containing 10 μg of TT/mL) in coating buffer (Na_2CO_3/$NaHCO_3$, pH=9.6) for 3 hours at 37 °C. Wells were first washed thrice with 200 μL/well of washing buffer and then blocked with 2% milk in washing fluid (for 1000 mL, 8 g NaCl, 1.45 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl and 0.05 % Tween-20) for 3 hours at 37°C. After single wash with 200 mUwell of washing buffer, the plates were incubated with various dilutions (1/50 to 1/6400) of the test serum. After an overnight incubation at 4 °C the plates were washed thrice with 200 μL/well of washing buffer and incubated with 100 μL of secondary antibody. When determining the amounts of IgG, IgA, or IgM, horse radish peroxidase (hrp) conjugated to the appropriate Anti-Ig was used. After a 3 hours incubation at 37 °C, the plates were washed thrice with 200 μL/well of washing buffer and the color was developed using o-phenyl diamine as hrp substrate. 0.4 mg/mL of o-phenyl-diamine in phosphate-citrate buffer (pH 4.5) with 0.4 pL H_2O_2 per mL was used for the purpose. After 2 minutes the reaction was stopped by the addition of 50 μL of 2N H_2SO_4. The absorbency was measured at 492 nm.

[0124] The method used to detect various isotypes was also ELISA based. It relied on the peroxidase-labeled, affinity purified secondary antibodies specific for IgG1 (1:1000), IgG2a (1:1000), IgG2b (1:1000), and IgG3 (1:200) which were all obtained from ICN ImmunoBiologicals. Further secondary antibodies included IgA (1:1000) and IgM (1:1000) linked to horse-radish peroxidase (Sigma, Neu-Ulm, Germany). The correspondingly labelled anti-mouse IgE was purchased from PharMingen (San Diego, CA. The antigens were again permitted to adsorb on test plates and incubated with the test serum after excess of the antigen had been washed away. Subsequently, 100 μL of appropriate specific secondary antibody solution was added to one of the six different plates, to determine anti-IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, respectively. The plates were incubated for 3 hours at 37 °C and processed further as described in previous paragraphs.

[0125] ***Challenge with antigen (the tetanus toxin) in vivo.*** On the day 35, test animals were challenged by injecting 50 times the LD_{50} of the tetanus toxin subcutaneously (s.c.). (The actual value of LD_{50} was fixed in separate experiments, during which a group of 16 weight-matched animals was challenged s.c. with increasing amounts of toxin and the number of survivors was determined.) To determine the acute TT toxicity in vaccinated animals, the clinical status

of such test mice was recorded for 4 days after the first challenge.

[0126] Non-protected mice showed signs of paralysis after 24 hours resulting in death, after 36 hours, at latest. Animals which developed no symptoms of paralysis or other anomaly over a 4 days period following the challenge were deemed immune against tetanus.

[0127] The long-term immunity was tested by challenging all immunised mice on a monthly basis with a dose of toxin corresponding to 50 times LD_{50}, for at least half a year.

Examples 1-2:

Aggregate size (stability) effect

[0128] Highly deformable vesicles (Transfersomes™: IDEA):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA, LA) relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0129] (Mixed lipid) Micelles:

65 mg phosphatidylcholine from soy bean (SPC)
35 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0130] Tetanus toxoid (2 mg/mL; Accurate Antibodies) used at the dose of
40 µg (20 µL) or 80 µg (40 µL) TT per mouse and immunisation

[0131] Application area: 1 cm^2 or 2 cm^2 for 40 µg or 80 µg TT per mouse on the upper dorsum.

[0132] To test the effect of formulation stability on the immunological properties of various, epicutaneously administered formulations, two kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) and relatively small micelles (diameter below 50 nm). The latter were chosen in the expectation that under sub-optimal conditions (owing to the lipid degradation or inappropriate aggregate composition) the latter may arise from the former.

[0133] **Antibody titres,** as reflected in the serum absorbency at 492 nm, are shown in figure 1. They show that mixed lipid micelles are less efficient antigen carriers than ultradeformable mixed lipid vesicles (Tfs) loaded with the same amount of TT. Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) were even less efficient immune response mediators.

[0134] **Animal protection data** reveal similar trend, as is seen in lower panel of figure 1.

Examples 3-4:

Aggregate deformability effect

[0135] Conventional lipid vesicles (liposomes):

100 mg phosphatidylcholine from soy bean (SPC)
0.4 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.5mL phosphate buffer, 10 mM, pH 6.5
2 mg/mL tetanus toxoid (Accurate Antibodies)

[0136] Highly deformable vesicles (Transfersomes™):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA) relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5

0.1 mL ethanol

[0137] Tetanus toxoid used at the dose of 40 µg or 80 µg TT/ mouse/ immunisation

[0138] Application area: 1 cm^2 or 2 cm^2 for 40 µg or 80 µg TT/ mouse/ immunisation on the upper dorsum.

[0139] **Results** obtained with the conventional vesicles differ from the data measured with highly deformable vesicles: simple liposomes, which do not cross the narrow pores in a barrier also do not elicit a substantial antibody titre. Conversely, the vesicles with a highly flexible and deformable, and thus better adaptable, membrane which were shown separately to move through the narrow pores in a barrier with greater ease, generate an appreciable quantity of antibody when applied on intact skin, according to the results of serum absorbency measurements (cf. figure 2).

Examples 5-10:

Antigen dose effect

[0140] Highly deformable vesicles:

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol
Tetanus toxoid (TT: Accurate Antibodies, New York, USA) concentration:
empty, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, giving raise to 0 µg, 10 µg, 20 µg, 40 µg or 80 µg TT/ mouse/ immunisation

[0141] Application area: 1 cm^2 for 0 µg, 10 µg, 20 µg, 40 µg and 2 cm^2 for 80 µg TT/mouse/ immunisation on the upper dorsum.

[0142] The results of this experimental series are illustrated in figure 3. It clearly shows the increase in immune response to epicutaneously administered tetanus toxoid in ultradeformable carriers with increasing TT dose. This is reflected in serum absorbency (up to the dose of 20 µg/immunisation), in specific antibody titre (up to the dose of 40 µg/immunisation), and in the survival data (which do not saturate for doses up to 80 µg/immunisation).

[0143] Less clarity is found in isotype distribution pattern, except for IgG1 (with a strong indication for the response saturation) and for IgG2b (perhaps, with the saturation between 40 µg and 80 µg per immunisation). IgM shows dose dependence similar to that of IgG1. The picture obtained for IgG2a is confusing.

Examples 11-13:

Antigen purity effect

[0144] Highly deformable vesicles:

as described with examples 5-10 (except in that the group treated with impure TT did not receive immunoadjuvant lipid A)

[0145] Tetanus toxoid: 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation

[0146] Application area: 2 cm^2 on the upper dorsum.

[0147] Antigen purity strongly affects the level of murire protection against tetanus toxin when the toxoid has been applied non-invasively on the skin. (Similar results obtained with injected antigen are not shown).

[0148] To substantiate the above mentioned statement" the medium filtrate from a culture of *Clostridium tetani* grown in vitro first was used as an impure antigen. To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times. Purified toxoid was purchased from Accurate Antibodies, NY, USA.

[0149] Swiss albino mice (n = 6) were immunised with identical nominal dose of impure antigen, with partially purified antigen supplemented with monophosphoryl lipid A or with purified antigen with monophosphoryl lipid A added. The antigen was always associated with similar Transfersomes. The composition and the method of preparation for the latter were the same are as described with previous examples. The details of immunisation schedule, bleeding times and challenge as well as analysis details were also similar to those mentioned before.

[0150] The results are given in figure 4. They demonstrate the role antigen purity plays in determining the quality as

well as the strength of an immune response against TT. The data shown in figure 4, moreover, indicate that the absorbency of even the specific antibody titre is not a reliable predictor of the therapeutic, that is, of prophylactic effect of an epicutaneous vaccination. This is due to the big differences in specific antibody isotypes which only contain a substantial proportion of Th1-like IgG2b compared to Th2-like IgG1 component if sufficiently pure antigen is used (see also page 12).

Examples 14-15 :

Comparison of epicutaneous and subcutaneous administration

[0151] Highly deformable vesicles, Transfersomes™ (IDEA):
as described with examples 5-10

[0152] Tetanus toxoid dose:

80 µg TT per epicutaneous immunisation
(using 2 mg TT/mL and application area of 2 cm^2)
40 µg TT per subcutaneous injection (using 2 mg TT/mL)

[0153] Using the same experimental procedures as described with examples 1-4, as appropriate, the antibody-specific serum titre, the level of animal protection against tetanus toxin and relative occurrence of different specific antibody isotypes was determined.

[0154] The results are given in figure 5. While the immunisation dependent increase in serum absorbency is comparable after invasive and non-invasive antigen administration the titre in the latter case is somewhat lower by the factor of 6 after primary immunisation and by the factor of 8 after second boost. Likewise, while the TT-specific levels of Th2-indicating IgG1 are similar in both arms of this experiment, the specific readings for other antibody subtypes, especially for IgG2a and at early time points also for IgG2b are by the factor 25 and 3, respectively, higher after antigen injections. However, the likelihood for the test mice to survive a subsequent challenge with a normally lethal dose of injected tetanus toxin is independent of the route of antigen administration, within the framework of this experimental series at least.

Examples 16-17:

Adjuvant skin treatment (pre-injection) effect

[0155] Highly deformable vesicles, Transfersomes™ (IDEA):

89.3 mg phosphatidylcholine from soy bean (SPC)
10.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (MLA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0156] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation of 6 Swiss albino mice per group using impure antigen

[0157] Application area: 2 cm^2 on the upper dorsum.

[0158] Transcutaneous transport of macromolecules associated with Transfersomes across the skin seems to be extremely gentle; it therefore fails to trigger the immune system toward Th2-like immune response, if the antigen is used in a low amount or is impure. To change the situation, the skin can be (pre)stimulated to release corresponding messenger molecules from the organ prior to the actual non-invasive antigen administration by means of ultradeformable vesicles. For this purpose we have pre-injected the application site with 0.1 mL of saline, or a mild formulation of non-antigenic vesicles, prepared from biodegradable material of similar composition as the antigen carrying vesicles one day before using the latter. For additional control, incomplete Freund's adjuvant was also injected in different animals 24 hours before the application of immuno-carriers on the skin.

[0159] Illustrative examples of results are given in figure 6. They reveal higher specific antibody titres, especially for and improved protection in the mice that were pre-treated by injections rather than carrier formulation on the skin, which served as a control. The effect of incomplete Freund's adjuvant is surprisingly weak.

[0160] It is noteworthy that the serum absorbency or the specific antibody titre and animal survival, that is protective vaccination effect, are not correlated.

Examples 18-21:

Low molecular weight adjuvant (lipid A) effect

[0161] Highly deformable immuno-modulated TT-Transfersomes™ (IDEA):

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.04 mol-% monophosphoryl Lipid A (LA)relative to SPC
0.9 mL phosphate buffer, 10 mM, pH 6.5
0.1 mL ethanol

[0162] Highly deformable standard immuno-vesicles, TT-Transfersomes™ (IDEA):
as above but without LA added

[0163] Tetanus toxoid: 2 mg/mL, with 20 µL or 40 µL corresponding to 40 µg or 80 µg TT per immunisation

[0164] Application area: 1 cm^2 or 2 cm^2, respectively, on the upper dorsum.

[0165] We believe that immuno-active, typically immunopotentiating, molecules must be present in the skin at the time of antigen presentation to the body by TT carriers that have crossed the barrier in order to achieve the desired immunological action of the antigen. To substantiate this conclusion we compared the outcome of non-invasive immunopresentation of TT by means of Transfersomes with or without a well known immunostimulant, monophosphoryl lipid A (LA), which is known to elicit generation of TNF in the body, for example. Two different antigen doses were used. In either case substantial titres and a measurable prophylactic immune response (partial immunity) was reached.

[0166] The absorbency of the serum increases as one would expect (cf. figure 7). Conversely, the effect of LA is better seen for the lower than for the higher dose used. This may be due to experimental variability or else reflect nonlinearity of dose vs. action curve for the typical immunisation data. It is possible, for example, that adjuvant is only efficient in the low dose range, whereas in the high dose regiment the system is quasi-saturated, leaving little possibility for the adjuvant to further enhance the immune response within the scope of experimental set-up. Complete animal protection against a normally lethal challenge with 50 LD_{50} was achieved in this test series with the higher TT dose in combination with LA only.

[0167] It was further observed that Th1-cytokine IgG2b was higher with LA groups, compared with the groups that received no LA. This difference was more pronounced for low doses, by the factor of 4, than for high doses, where only an enhancement by the factor of 2 was observed. Th2-cytokine IgG1 was present predominantly, except in the low dose with LA group in which IgG2b contributed comparably.

Examples 22-23:

High molecular weight adjuvant, IL-12 cytokine effect

[0168] Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-10, plus
0.01 mg IL-12 per mL immunogen suspension

[0169] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation (partially purified as described with examples 9-11)

[0170] Application area: 2 cm^2 on the upper dorsum of Swiss albino mice.

[0171] To study the effect of cytokines on results of non-invasive, epicutaneous vaccination with tetanus toxoid, a combination of monophosphoryl lipid A with 0.4 µg IL-12 per mouse was used. 80 µg of IL-12 was administered per mouse in association with Transfersomes loaded with tetanus toxoid and monophosphoryl lipid A. The details of immunisation schedule, bleeding intervals, or the final challenge with the tetanus toxin were the same as mentioned above.

[0172] The results of experimental series are illustrated in figure 8. The corroborate the conclusion that the presence of pro Th2 cytokines in the skin during the course of immunopresentation following an epicutaneous TT administration positively affects the outcome of vaccination. This is seen in serum absorbency, the specific antibody titre as well as in the test animal survival probability.

[0173] The effect discussed with examples 22-23 was verified by incorporating cytokines other than IL-12 into immunogen formulation. The results are shown in figure 9.

Examples 24-25:

High molecular weight adjuvant (IFN-γ and GM-CSF + IL-4) effect

[0174] Highly deformable vesicles, Transfersomes™ (IDEA):

as described with examples 5-8, plus
0.05 mg IFN-γ and 0.004 mg GM-CSF and 0.004 mg IL-4 per mL immunogen suspension

[0175] Tetanus toxoid, 2 mg/mL, corresponding to 80 µg TT per mouse/ immunisation (impure)
[0176] Application area: 2 cm^2 on the upper dorsum of Swiss albino mice.
[0177] The effect discussed with examples 22-23 was confirmed also with a different blend of cytokines. The results are shown in figure 10.

Examples 28-29:

Booster effect (maturation of immune response)

[0178] In most of previous examples, a consistent pattern was observed whenever the absorbency was measured during the time course of immunisation. The immune response increased with each boost, compared to the response obtained after primary immunisation (see Figures 3, 4, 5, 6, 7, 8). The primary response was characterised by predominance of IgM, followed by gradual appearance of IgG after the first boost and by the appearance of even grater amounts of IgG after the second boost, with a concurrent disappearance of IgM. This typical pattern of isotype signifies affinity maturation in the immune response. During the process, the average affinity of a mixture of specific antibodies increases with repeated immunisations.
[0179] Results of various epicutaneous vaccination experiments suggest that it may be advantageous to combine an invasive priming vaccination with non-invasive secondary (boost) immunisation.

Examples 30-72:

Release of cytokines from the skin in vitro by Transfersomes

[0180] Highly deformable vesicles (Transfersomes type C):

87.4 mg phosphatidylcholine from soy bean (SPC)
12.6 mg sodium cholate (NaChol)
0.9 mL phosphate buffer, 50 mM, pH 7.3
2.5 µL thereof

[0181] Highly deformable vesicles (Transfersomes type T):

50 mg phosphatidylcholine from soy bean (SPC)
50 mg polysorbate (Tween 80)
0.9 mL phosphate buffer, 50 mM, pH 6.5
2.5 µL thereof

[0182] Positive control A:
2.5 pL 5% sodium dodecylsulphate (SDS)
[0183] Positive control B:
100 µL lipopolysaccharide (LPS; 10^5 U/mL)
[0184] Negative control:
2.5 pL of phosphate buffered saline (PBS)
[0185] All products were tested undiluted.
[0186] Cell type: Normal human keratinocytes, forming a pluri-stratified epithelium with a compact stratum corneum were used; histology revealed strong resemblance with human epidermis in vivo.
[0187] Method: Keratinocytes were inoculated on polycarbonate filter inserts of 0.63 cm^2 in chemically defined, supplemented medium, and cultured for 17 days at the air-liquid interface.
[0188] Test measurements: given amount of each tested product was deposited with a micropipette and spread

evenly over the surface of the stratum corneum of eight reconstituted epidermis using a small sterile device. The cultures were incubated at 37 °C, 5 % CO_2 for 24 hours. Quadruplicate cultures (except for the LPS treated cells which were incubated in duplicate) were washed with 0.5 mL of PBS and incubated on 300 µL of 0.5 mg/mL MTT for 3 hours at 37 °C, 5 % CO_2.

[0189] The release of inflammatory mediators (IL1α, IL2, IL4, IL8, IL10, IFN-γ, and TNF-α) in the medium underlying the tissues was quantified using ELISA kits (R&D systems UK; Quantikine), specific for each type of immuno-modulator to be measured.

	IL1-α (pg/mL) Mean +/- SD	IL8 (pg/mL) Mean +/- SD	TNF-α (pg/mL)
Negative control (PBS, n=2)	5.1 +/- 0.5	<31	not detectable
Positive control A (SDS 5 %, n=2)	**314.2 +/- 6.1**	**147.5 +/- 32**	not detectable
Positive control B (LPS, n=1)	32.0	**5161**	**113.4**
Transfersomes C (02-05, n=2)	12.3 +/- 0.9	68.3 +/- 16.8	not detectable
Transfersomes T (TT0009/175, n=2)	11.7 +/- 1.2	50.8 +/- 14.0	not detectable
Transfersomes O (TT0017/15, n=2)	**185.5 +/- 170.1**	58.4 +/- 27.0	not detectable
The relatively big standard deviation observed with Transfersomes O can be explained by the fact that the product was difficult to spread uniformly onto the stratum corneum of the reconstructed epidermis.			

[0190] TNF-α level was increased to the level of 113.43 pg/mL when the cells were in contact with the positive controls containing LPS, which is an established immunoadjuvant.

[0191] IL8 concentration after cells incubation with Transfersomes exceeded the lower limit of detection by just the factor of 2, which in one case is not and in the other is barely significant at 95 % confidence level, but in either situation is negligible compared to the increase observed with the positive control containing the immunoadjuvant LPS, which gave a 167x higher value.

[0192] Non-specific irritant, SDS, released a great quantity of IL-1α from the skin cells into the bathing medium in vitro. The possibility exists, that an amount of comparable quantity was released from the cells incubated with Transfersomes O, comprising the potentially irritating oleic acid at a high concentration, but firm conclusion is prevented by the great standard deviation in the results obtained with the latter test system.

[0193] IL-1α concentration for the other tested Transfersomes of type A and type B changed to approximately 2 times the background level. This difference is statistically significant, compared to negative controls, but practically negligible, taken that the increase observed with the positive control containing LPS was more than 60 times higher.

[0194] IFN-γ, IL-2, IL4 or IL10 was not elevated to a measurable level, suggesting a lack of release of these cytokines, under any other test condition.

[0195] Taken together the above mentioned findings suggest that Transfersomes do not release cytokines or induce the generation of such molecules from the skin cells. This explains the need for using immunoadjuvants/modulators when antigens or allergens are to be delivered across the skin with such carriers and elicit a therapeutic or prophylactic immune response.

Examples 73-82:

Bacterial wall component, cholera toxin, as specific immuno-adjuvant:

[0196] Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)
13.7 mg sodium cholate (NaChol)
0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera toxin (CT; Sigma, Neu-Ulm), 10 µg/immunisation plus, if required,
Tetanus toxoid (TT, pure; Accurate antibodies) 2 mg/mL,

[0197] Volume doses corresponding to 0 µg TT/mouse/immunisation (negative control), 1 µg TT/mouse, 5 µg TT/mouse, 10 µg TT/ mouse, 20 µg TT/ mouse, 40 µg TT/mouse (in the case of CT usage) and 80 µg TT/mouse (without CT) were used epicutaneously over an area of up to 2 cm^2 on the upper dorsum of 4-6 Swiss albino mice; 20 µg TT/ mouse/immunisation were injected subcutaneously at the corresponding site in the positive control group. Unimmunised mice were used as another negative control.

[0198] The protective effect of epicutaneous antigen administration was excellent when cholera toxin was included into the test formulation in combination with the tetanus toxoid. Formulation without this immunoadjuvant yielded inferior protection, as demonstrated by the fact that 1 animal out of 4 (25 %) was paralytic after the challenge with tetanus toxin.

[0199] The results shown in figure 10 reveal that the antigen doses in excess of 20 µg/immunisation ensured complete protection, which was not the case with the other tested adjuvants or adjuvant treatments (see previous examples). Lower dosage of antigen gave qualitatively similar effect but was insufficient to guarantee the survival of all test mice, except in the test group which received 5 µg TT/immunisation. (This implies that TT doses between 1 µg/immunisation and 15 µg/immunisation belong to the transition region.) Other doses of cholera toxin might be equally or even more beneficial, however.

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Claims

1. A transdermal vaccine comprising

   (a) a transdermal carrier which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance, and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains;
   (b) a compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity itself; and
   (c) an antigen or an allergen.

2. The vaccine according to claim 1, wherein the compound displaying or inducing cytokine or anti-cytokine activity and the antigen are associated with the penetrant.

3. The vaccine according to any one of claims 1 or 2, wherein the less soluble self-aggregating molecule is a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

4. The vaccine according to any one of claims 1 to 3, wherein the average diameter of the penetrant is between 30 nm and 500 nm, preferably between 40 nm and 250 nm, even more preferably between 50 nm and 200 nm and particularly preferably between 60 nm and 150 nm.

5. The vaccine according to any one of claims 1 to 4, wherein the total weight of droplets in the formulation for the use on human or animal skin is 0.01 weight-% (w-%) to 40 weight-% of total mass, in particular between 0.1 w-% and 30 w-%, and most preferably between 5 w-% and 20 w-%.

6. The vaccine according to any one of claims 1 to 5, wherein total antigen concentration is between 0.001 and 40 w-% of the total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 20 w-% and most preferably between 0.5 w-% and 10 w-%.

7. The vaccine according to any one of claims 1 to 6 further comprising

   (da) a low molecular weight chemical irritant; and/or
   (db) an extract or a compound from a pathogen or a fragment or a derivative thereof.

8. The vaccine according to any one of claims 1 to 7 wherein the compound exerting cytokine activity is IL-4, IL-2, TGF, IL-6, TNF, IL-1α and IL-1β, a type I interferon, preferably IFN-alpha or IFN-β, IL-12, IFN-γ, TNF-β, IL-5 or IL-10.

9. The vaccine according to any one of claims 1 to 8 wherein the compound displaying anti-cytokine activity is an anti-cytokine antibody or the corresponding active fragment, a derivative or an analogue thereof.

10. The vaccine according to any one of claims 1 to 9 wherein the antigen is derived from a pathogen.

11. The vaccine according to claim 10 wherein said pathogen is selected from extracellular bacteria, including pus-forming cocci, such as *Staphylococcus* and *Streptococcus,* gram-negative bacteria, such as Meningococcus and Gonococcus species, species of *Neisseria,* gram negative bacteria, including enteric organisms such as *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis,* and gram-positive bacteria (e.g. *Bacillus pestis, BCG*), particularly anaerobes, such as the *Clostridium* species, bacteria and viruses, which survive and replicate

within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes,* retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpex viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases and pathogens that cause various neoplasiae, auto-immune diseases or are related to other pathological states of the animal or human body which do not necessarily result from pathogen infections.

12. The vaccine according to any one of claims 1 to 11, wherein the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

13. The vaccine according to any of claims 1 to 12, wherein the concentration of each compound displaying cytokine activity used is selected to be up to 1000 times higher than the concentration optimum established in the corresponding tests with the antigen dose and immunoadjuvant chosen, performed by injecting the formulation or performing the tests *in vitro,* and preferably is up to 100x, more often up to 50x and even better up to 20x higher.

14. The vaccine according to any one of claims 7 to 13, wherein the pathogen extract or compound is a lipopolysaccharide, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a pathogen; an extract of a pathogen, including bacterial exo- and endotoxins, preferably cholera toxin and the heat labile toxin of *E. coli,* an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, or a purified protein derivative of *M. tuberculosis,* LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes,* or outer membrane protein of group B *Neisseria meningitidis* (GBOMP).

15. The vaccine according to claim 14 wherein said lipopolysaccharide is lipid A or a derivative and modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose.

16. The vaccine according to any one of claims 7 to 13, wherein the concentration of the pathogen compound derived from a pathogen is between 10x lower and up to 1000x higher than that otherwise used with the corresponding injected formulations employing similar antigen, the epicutaneously administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

17. The vaccine according to any one of claims 7 to 16 wherein said low molecular weight irritant is selected from the classes of allergenic metal ions, acids, bases, irritating fluids, (fatty-) alcohols, (fatty-) amines, (fatty-) ethers, (fatty-) sulphonates, -phosphates, etc., or other suitable solvents or amphiphiles, or from the group of surfactant-like molecules, often with the skin permeation enhancing capability, as well as derivatives or combinations thereof.

18. The vaccine according to any one of claims 7 to 17, wherein the concentration of a low molecular weight irritant is chosen to be by at least the factor of 2, more often by the factor of 5, and even better by the factor of 10 or more, below the concentration which in independent tests on the same or a comparable subject is deemed to be unacceptable owing to the local irritancy, as assessed by the methods and standards commonly used to test such an irritant.

19. The vaccine according to any one of claims 7 to 16 wherein the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., a part of implantation material.

20. The vaccine according to any one of claims 1 to 19, wherein the applied dose of an antigen differs by the factor of 0.1 to 100 from the dose which otherwise would have to be injected in the process of immunisation, but more often is in the range between 0.5 to 50, even better between 1 and 20 and ideally is less than 10x higher than that used with an injection.

21. The vaccine according to any one of claims 1 to 20, wherein the applied penetrant dose is between 0.1 mg cm^{-2} and 15 mg cm^{-2}, even more often is in the range 0.5 mg cm^{-2} and 10 mg cm^{-2}, and preferably is between 1 mg cm^{-2} and 5 mg cm^{-2}.

22. The vaccine according to any one of claims 1 to 21 wherein said antigen is a pure or purified antigen.

23. A kit comprising, in a bottled or otherwise packaged form, at least one dose of the vaccine according to any one of claims 1 to 22.

24. The kit according to claim 23 further comprising at least one injectable dose of the antigen specified in claim 11 or of the allergen specified in claim 12.

25. Use of a vaccine according to any one of claims 1 to 22 for the preparation of a pharmaceutical composition for generating a protective immuno response in a mammal.

26. The use according to claim 25 wherein different treatment areas are selected to control the appiled immunogen dose and the outcome of therapeutic vaccination.

27. The use according to claim 25 or 26, wherein a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the administration of resulting formulation on the skin.

28. The use according to any one of claims 25 to 27, wherein the vaccine according to any one claims 1 to 22 is to be applied on the skin after pre-treating the organ by an immunoadjuvant manipulation, said manipulation comprising, for example, skin rubbing, pressing, heating, exposing to an electrical or mechanical, e.g. ultrasound field, etc., or injecting a non-immunogenic formulation in the skin, provided that any such treatment releases immunoadjuvant compounds from the skin or other peripheral immuno-active tissues or else reduces the concentration / duration of action of antagonists to the desired vaccination.

29. The use according to any one of claims 25 to 28 wherein the immunogen is to be applied in a non-occlusive patch.

30. The use of any one of claims 25 to 29 **characterised in that** at least one dose of vaccine is to be administered.

31. The use according to claim 30 wherein said vaccine is to be administered as a booster vaccination.

32. The use according to claim 31, wherein the primary immunisation is done invasively, typically using a subcutaneous injection or some other suitable skin barrier perforating/destructing method, and wherein the at least one subsequent, booster immunisation is to be done non-invasively.

33. The use according to any one of claims 25 to 32, wherein the vaccine is to be applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immunotolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.

34. The use according to claim 33, wherein the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.

35. The use according to any one of claims 25 to 34, wherein the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is to be determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

36. Use of the transdermal carrier, the compound which specifically releases or specifically induces cytokine or anti-cytokine activity or exerts such an activity, the antigen or allergen, and optionally an extract or a compound from a microorganism or a fragment or a derivative thereof, and/or a low molecular weight chemical irritant as defined in any one of the preceding claims for the preparation of a vaccine for inducing a protective or tolerogenic immune response.

Patentansprüche

1. Transdermaler Impfstoff, umfassend

   (a) einen transdermalen Träger, der ein Durchdringungsmittel ist, das in einem wäßrigen Lösungsmittel suspendiert oder dispergiert ist, in der Form eines winzigen Flüssigkeitstropfens, der von einer membranähnlichen Hülle aus einer oder mehreren Schichten von mindestens zwei verschiedenen Stoffen oder zwei verschiedenen Formen eines Stoffes, mit der Tendenz zu aggregieren umgeben ist, wobei die Stoffe oder Formen eines Stoffes sich mindestens um den Faktor 10 in der Löslichkeit in einem vorzugsweise wäßrigen, flüssigen Medium unterscheiden, so daß der mittlere Durchmesser von Homoaggregaten des löslicheren Stoffes oder Form des Stoffes oder der mittlere Durchmesser der Heteroaggregate bestehend aus beiden Stoffen oder Formen des Stoffes kleiner ist als der mittlere Durchmesser der Homoaggregate des weniger löslichen Stoffes oder Form des Stoffes, und/oder wobei die löslichere Komponente die Tendenz hat, den durchdringenden Tropfen zu solubilisieren, und wobei der Gehalt einer solchen Komponente bis zu 99 mol-% der Konzentration beträgt, die zum Solubilisieren des Tropfen erforderlich ist, oder ansonsten bis zu 99 mol-% der Sättigungskonzentration in dem nicht solubilisierten Tropfen entspricht, was auch immer höher ist, und/oder wobei die elastische Deformationsenergie des Tropfens, der die membranähnliche Hülle umgibt, mindestens fünffach niedriger ist, vorzugsweise mindestens zehnfach niedriger und idealerweise mehr als zehnfach niedriger ist als diejenige von roten Blutzellen oder von Phospholipid-Doppelschichten mit flüssigen, aliphatischen Ketten;
   (b) eine Verbindung, die spezifisch Cytokin- oder Anti-Cytokin-Aktivität freisetzt oder induziert oder selbst solch eine Aktivität zeigt; und
   (c) ein Antigen oder ein Allergen.

2. Impfstoff nach Anspruch 1, wobei die Verbindung, die Cytokin- oder Anti-Cytokin-Aktivität aufweist oder induziert, und das Antigen mit dem Durchdringungsmittel verknüpft sind.

3. Impfstoff nach einem der Ansprüche 1 oder 2, wobei das weniger lösliche, selbstaggregierende Molekül ein polares Lipid ist und die löslichere Komponente ein grenzflächenaktiver Stoff oder ein grenzflächenaktives Stoffähnliches Molekül ist oder ansonsten diejenige Form eines polaren Lipides, die genügend löslich für den Zweck der Erfindung ist.

4. Impfstoff nach einem der Ansprüche 1 bis 3, wobei der mittlere Durchmesser des Durchdringungsmittels zwischen 30 nm und 500 nm ist, vorzugsweise zwischen 40 nm und 250 nm, bevorzugt zwischen 50 nm und 200 nm und besonders bevorzugt zwischen 60 nm und 150 nm ist.

5. Impfstoff nach einem der Ansprüche 1 bis 4, wobei das Gesamtgewicht der Tropfen in der Formulierung zum Gebrauch auf menschlicher oder tierischer Haut 0,01 Gewichtsprozent bis 40 Gewichtsprozent der gesamten Masse, insbesondere zwischen 0,1 Gewichtsprozent und 30 Gewichtsprozent und vorzugsweise zwischen 5 Gewichtsprozent und 20 Gewichtsprozent ist.

6. Impfstoff nach einem der Ansprüche 1 bis 5, wobei die Gesamtantigenkonzentration zwischen 0,001 und 40 Gewichtsprozent der gesamten durchdringenden Masse ist, im besonderen zwischen 0,01 Gewichtsprozent und 30 Gewichtsprozent, besser zwischen 0,1 Gewichtsprozent und 20 Gewichtsprozent und am meisten bevorzugt zwischen 0,5 Gewichtsprozent und 10 Gewichtsprozent ist.

7. Impfstoff nach einem der Ansprüche 1 bis 6, ferner umfassend

   (da) ein chemisches Reizmittel mit niedrigem Molekulargewicht; und/oder
   (db) einen Extrakt oder eine Verbindung aus einem Pathogen oder ein Fragment oder ein Derivat davon.

8. Impfstoff nach einem der Ansprüche 1 bis 7, wobei die Cytokin-Aktivität aufweisende Verbindung IL-4, IL-2, TGF, IL-6, TNF, IL-1α und IL-1β, ein Typ 1-Interferon, vorzusweise IFN-α oder IFN-β, IL-12, IFN-γ, TNF-β, IL-5 oder IL-10 ist.

9. Impfstoff nach einem der Ansprüche 1 bis 8, wobei die Anti-Cytokin-Aktivität aufweisende Verbindung ein Anti-Cytokin-Antikörper oder das entsprechende aktive Fragment, ein Derivat oder ein Analog davon ist.

10. Impfstoff nach einem der Ansprüche 1 bis 9, wobei das Antigen von einem Pathogen stammt.

11. Impfstoff nach Anspruch 10, wobei das Pathogen ausgewählt ist aus extrazellulären Bakterien, einschließlich eiterbildenden Kokken, wie *Staphylococcus* und *Streptococcus*, gram-negativen Bakterien, wie Meningococcus und Gonococcus-Arten, *Neisseria*-Arten, gram-negativen Bakterien, einschließlich Darmorganismen wie *E. coli, Salmonella, Shigella, Pseudomonas, Diptheria, Bordetella Pertussis*, und gram-positiven Bakterien (z.B. *Bacillus pestis, BCG*), besonders anaeroben, wie die *Clostridium*-Arten, Bakterien und Viren, die innerhalb von Wirtszellen überleben und sich replizieren, umfassend Mycobakterien (z.B. *M, tuberculosis*) und *Listeria monocytogenes*, Retroviren und Adenoviren, einschließlich Hepatitisvirus, (menschlicher) Immundefizienzvirus, Herpesviren, Pocken- (Winpocken), Influenza-, Masern-, Mumps- und Polio-Viren, Cytomegalovirus, Rhinovirus, usw., und Pilzen, die innerhalb von Wirtszellen gedeihen, Parasiten, einschließlich tierischen Parasiten, wie Protozoen und Helminthen, und Ectoparasiten, wie Zecken und Milben, oder *Brucella*-Arten, einschließlich des Cholera verursachenden Agens, Haemophilus-Arten, ebenso wie Pathogenen, die Parathyphus, Pest, Tollwut, Tetanus und Röteln auslösen, und Pathogenen, die verschiedene Neoplasien, Autoimmunkrankheiten oder die mit anderen pathologischen Zuständen des tierischen oder menschlichen Körpers verwandt sind, verursachen, die nicht notwendigerweise von pathogenen Infektionen herrühren.

12. Impfstoff nach einem der Ansprüche 1 bis 11, wobei das Allergen xenogenen oder endogenen Ursprungs ist, von einem Mikroorganismus, einem Tier oder einer Pflanze stammt, oder zu der Gruppe künstlicher und/oder reizender inorganischer Stoffe gehört, oder zu solchen Teilen oder Komponenten des menschlichen Körpers gehören, die fälschlicherweise durch das Körperimmunsystem prozessiert oder dem Körperimmunsystem exponiert wurden.

13. Impfstoff nach einem der Ansprüche 1 bis 12, wobei die-Konzentration jeder verwendeten Verbindung, die Cytokin-Aktivität aufweist, bis zu 1000-fach höher gewählt ist als das Konzentrationsoptimum, das in den entsprechenden Versuchen mit der Antigendosis und dem gewählten Immunadjuvanz etabliert wurde, durchgeführt mittels Injektion der Formulierung oder durch in vitro-Versuche, und vorzugsweise bis zu 100-fach, öfters bis zu 50-fach und noch besser bis zu 20-fach höher ist.

14. Impfstoff nach einem der Ansprüche 7 bis 13, wobei der Pathogenextrakt oder die Verbindung ein Lipopolysaccharid, Cordfactor (Trehalose-Dimycolat), Muramyldipeptid, oder ein anderes (Poly)saccharid oder (Poly) peptid identisch mit oder ähnlich einem immunologisch aktiven Teil einer Membran eines Pathogens ist; ein Extrakt eines Pathogens, einschließlich bakteriellen Exo- und Endotoxinen, vorzugsweise Choleratoxin und das hitzelabile Toxin von *E. coli*, ein A-Kette-Derivat, eine Komponente mit einer ADP-ribosylierenden Aktivität, ein Peptidoglycan, ein von Clostridium stammendes Toxin, oder ein gereinigtes Proteinderivat von *M. tuberculosis*, LT-R192G, Fibronectin bindendes Protein 1 von *Streptococcus pyrogenes*, oder ein äußeres Membranprotein von Gruppe B-*Neisseria meningitidis* (GBOMP).

15. Impfstoff nach Anspruch 14, wobei das Lipopolysaccharid Lipid A oder ein Derivat ist und Modifikationen davon, wie Monophosphoryl-Lipid A, oder sein Analog, wie ein Fettderivat von Saccharose.

16. Impfstoff nach einem der Ansprüche 7 bis 13, wobei die Konzentration der Pathogenverbindung, die von einem Pathogen stammt, zwischen 10-fach niedriger und bis zu 1000-fach höher ist als diejenige, die sonst mit den entsprechenden injizierten Formulierungen unter Verwendung eines ähnlichen Antigens verwendet wird, wobei die epikutan verabreichte Immunadjuvantkonzentration sich öfter von der injizierten Immunadjuvantkonzentration durch den Faktor zwischen 0,5 und 100 unterscheidet, oder besser, durch den Faktor zwischen 1 und 50 und im besten Fall zwischen 2 und 25.

17. Impfstoff nach einem der Ansprüche 7 bis 16, wobei das Reizmittel mit niedrigem Molekulargewicht ausgewählt ist aus den Klassen von allergenen Metallionen, Säuren, Basen, reizenden Flüssigkeiten, Fettalkoholen, Fettaminen, Fettäthern, Fettsulfonaten, Fettphosphaten, usw., oder anderen geeigneten Lösemitteln oder Amphiphilen, oder aus der Gruppe von grenzflächenaktiven Stoff-ähnlichen Molekülen, oft mit die Hautdurchdringung verstärkenden Eigenschaften, sowie Derivaten oder Kombinationen davon.

18. Impfstoff nach einem der Ansprüche 7 bis 17, wobei die Konzentration des Reizmittels mit niedrigem Molekulargewicht um mindestens den Faktor 2, öfters um den Faktor 5, besser um den Faktor 10 oder mehr, unterhalb der Konzentration gewählt ist, die in unabhängigen Versuchen am selben oder an einem vergleichbaren Subjekt auf Grund der lokalen Reizung als nicht akzeptabel angesehen wird, ermittelt durch die Methoden und Standards, die gewöhnlich zur Untersuchung eines solchen Reizmittels verwendet werden.

19. Impfstoff nach einem der Ansprüche 7 bis 16, wobei das Allergen zu der Klasse von Inhalationsallergenen gehört,

einschließlich, aber nicht beschränkt auf verschiedene Pollen, Sporen, Stückchen von Tierhaar, Haut, Feder, natürlichen und synthetischen Textilien, Weizen, (Haus)-Staub, einschließlich Milben, ferner Nahrungsmittel- und Medikamentenallergene, Kontaktallergene, Injektions-, Invasions- oder Depotallergene, wie verschiedene (gastrointestinale) Würmer, Echinokokken, Trichinen, usw., ein Teil eines Implantationsmaterials.

20. Impfstoff nach einem der Ansprüche 1 bis 19, wobei die verabreichte Dosis eines Antigens sich um den Faktor 0,1 bis 100 von der Dosis, die sonst im Verfahren der Immunisierung injiziert worden wäre, unterscheidet, aber öfters im Bereich zwischen 0,5 bis 50, besser zwischen 1 und 20, und idealerweise weniger als 10-fach höher ist als diejenige, die mit einer Injektion verwendet wird.

21. Impfstoff nach einem der Ansprüche 1 bis 20, wobei die verabreichte durchdringende Dosis zwischen 0,1 mg/cm^2 und 15 mg/cm^2, öfters im Bereich 0,5 mg/cm^2 und 10 mg/cm^2, und vorzugsweise zwischen 1 mg/cm^2 und 5 mg/cm^2 ist.

22. Impfstoff nach einem der Ansprüche 1 bis 21, wobei das Antigen ein reines oder gereinigtes Antigen ist.

23. Kit, umfassend mindestens eine Dosis des Impfstoffes nach einem der Ansprüche 1 bis 22, in Flaschen- oder anderweitig verpackter Form.

24. Kit nach Anspruch 23, weiterhin umfassend mindestens eine injizierbare Dosis des in Anspruch 11 definierten Antigens oder des in Anspruch 12 definierten Allergens.

25. Verwendung eines Impfstoffes nach einem der Ansprüche 1 bis 22 für die Herstellung eines Arzneimittels zur Erzeugung einer schützenden Immunantwort in einem Säuger.

26. Verwendung nach Anspruch 25, wobei verschiedene Behandlungszonen ausgewählt werden, um die verabreichte Immunogendosis und das Ergebnis der therapeutischen Impfung zu kontrollieren.

27. Verwendung nach den Ansprüchen 25 oder 26, wobei eine Suspension von antigenfreien Durchdringungsmitteln mit dem Antigen beladen wird, um sie mit dem Antigen am Tag vor der Verabreichung zu verbinden, vorzugsweise 360 Minuten, bevorzugter 60 Minuten, und noch mehr bevorzugt 30 Minuten vor der Verabreichung der entstandenen Verbindung auf die Haut.

28. Verwendung nach einem der Ansprüche 25 bis 27, wobei der Impfstoff nach einem der Ansprüche 1 bis 22 auf die Haut verabreicht wird, nachdem das Organ durch eine Immunadjuvantmanipulation vorbehandelt worden ist, wobei die Manipulation z.B. darin besteht, daß man die Haut reibt, drückt, erwärmt, einem elektrischen oder mechanischen, z.B. Ultraschall-Feld aussetzt, usw., oder das Injizieren einer nicht immunogenen Formulierung in die Haut, mit der Maßgabe, daß jede dieser Behandlungen Immunadjuvantverbindungen aus der Haut oder anderen peripheren, immunaktiven Geweben freisetzt, oder sonst die Konzentration/Dauer der Wirkung von Antagonisten auf die gewünschte Impfung reduziert.

29. Verwendung nach einem der Ansprüche 25 bis 28, wobei das Immunogen in einem nicht-okklusiven Pflaster verabreicht wird.

30. Verwendung nach einem der Ansprüche 25 bis 29, **dadurch gekennzeichnet, daß** mindestens eine Dosis des Impfstoffes verabreicht wird.

31. Verwendung nach Anspruch 30, wobei der Impfstoff als eine Booster-Impfung verabreicht wird.

32. Verwendung nach Anspruch 31, wobei die primäre Immunisierung invasiv vorgenommen wird, typischer Weise durch subkutante Injektion oder eine andere geeignete, die Hautbarriere durchdringende/zerstörende Methode, und wobei mindestens eine folgende Booster-Immunisierung nicht invasiv vorgenommen wird.

33. Verwendung nach einem der Ansprüche 25 bis 32, wobei der Impfstoff zwischen 2 und 10, vorzugsweise zwischen 2 und 7, bevorzugt bis zu 5 und am bevorzugtesten bis zu 3 mal verabreicht wird, wenn ein nicht-allergenes Antigen verwendet wird, oder im Falle eines Allergens, so oft vorgenommen wird, wie erforderlich ist, um die gewünschte Immuntoleranz zu erreichen, festgestellt durch ein geeignetes Bestimmungsverfahren, oder sonst, den Versuch als gescheitert anzusehen.

34. Verwendung nach Anspruch 33, wobei das Zeitintervall zwischen den aufeinander folgenden Impfungen zwischen 2 Wochen und 5 Jahren gewählt ist, öfters zwischen 1 Monat und bis zu 3 Jahren, häufiger zwischen 2 Monaten und 1,5 Jahren.

35. Verwendung nach einem der Ansprüche 25 bis 34, wobei der Fluß der Durchdringungsmittel, die ein Immunogen durch die verschiedenen Poren in einem definierten Hindemis tragen, als eine Funktion einer geeigneten Antriebskraft oder eines Druckes, die oder der über das Hindernis hinweg wirken, bestimmt wird, und die Daten dann in geeigneter Weise über eine charakteristische Kurve beschrieben werden, die wiederum dazu verwendet wird, die Formulierung oder Anwendung weiter zu optimieren

36. Verwendung des transdermalen Trägers, der Verbindung, die spezifisch Cytokin- oder Anti-Cytokin-Aktivität induziert oder freisetzt oder solche Aktivität zeigt, des Antigens oder Allergens, und gegebenenfalls eines Extrakts oder einer Verbindung eines Mikroorganismus oder eines Fragments oder eines Derivats davon, und/oder eines chemischen Reizmittels mit niedrigem Molekulargewicht, gemäß der Definition in einem der vorangegangenen Ansprüche zur Herstellung eines Impfstoffes zur Induktion einer schützenden oder toleranten Immunantwort.

Revendications

1. Vaccin transdermique comprenant

   (a) un porteur transdermique qui est pénétrant, en suspension ou dispersé dans un solvant aqueux, sous la forme d'une minuscule gouttelette de liquide entourée d'un enrobage de type membranaire d'une ou plusieurs couches d'au moins deux substances différentes ou deux formes différentes d'une substance ayant tendance à s'agréger, lesdites substances ou formes d'une substance différant d'au moins un facteur 10 en terme de solubilité dans un milieu liquide, de préférence aqueux, de sorte que le diamètre moyen d'homo-agrégats de la substance ou forme de la substance plus soluble ou le diamètre moyen d'hétéro-agrégats comprenant lesdites deux substances ou formes de ladite substance est inférieur au diamètre moyen d'homo-agrégats de la substance ou forme de la substance moins soluble, et/ou dans lequel le composant plus soluble a tendance à solubiliser la gouttelette pénétrante et dans lequel la teneur d'un tel composant peut atteindre 99 % molaire de la concentration requise pour solubiliser la gouttelette ou sinon correspond jusqu'à 99 % molaire de la concentration de saturation dans la gouttelette non solubilisée, suivant le plus élevé, et/ou dans lequel l'énergie de déformation élastique de la gouttelette entourant le revêtement de type membranaire est au moins 5 fois plus faible, plus préférablement est au moins 10 fois plus faible et idéalement est plus de 10 fois plus faible que celle de globules rouges ou des bicouches de phospholipides comprenant des chaînes aliphatiques fluides ;
   (b) un composé qui libère spécifiquement ou induit spécifiquement l'activité cytokine ou anticytokine ou exerce une telle activité lui-même ; et
   (c) un antigène ou un allergène.

2. Vaccin selon la revendication 1, dans lequel le composé présentant ou induisant l'activité cytokine ou anticytokine et l'antigène sont associés au pénétrant.

3. Vaccin selon l'une quelconque des revendications 1 à 2, dans lequel la molécule auto-agrégante moins soluble est un lipide polaire et le composant plus soluble est un agent tensioactif ou une molécule de type tensioactif ou sinon une forme de lipide polaire telle qu'il soit suffisamment soluble pour les besoins de l'invention.

4. Vaccin selon l'une quelconque des revendications 1 à 3, dans lequel le diamètre moyen du pénétrant est compris entre 30 nm et 500 nm, de préférence entre 40 nm et 250 nm, encore plus préférablement entre 50 nm et 200 nm et de la manière la plus préférée entre 60 nm et 150 nm.

5. Vaccin selon l'une quelconque des revendications 1 à 4, dans lequel le poids total des gouttelettes dans la formule pour utilisation sur la peau humaine ou animale est de 0,01 % en poids (% m/m) à 40 % en poids de la masse totale, en particulier entre 0,1 % m/m et 30 % m/m et le plus préférablement entre 5% m/m et 20% m/m.

6. Vaccin selon l'une quelconque des revendications 1 à 5, dans lequel la concentration totale de l'antigène est de 0,001 et 40 % m/m de la masse totale pénétrante, en particulier entre 0,01 % m/m et 30 % m/m, plus préférablement entre 0,1 % m/m et 20 % m/m et le plus préférablement entre 0,5 % m/m et 10 % m/m.

7. Vaccin selon l'une quelconque des revendications 1 à 6 comprenant en outre

(da) un irritant chimique à bas poids moléculaire; et/ou
(db) un extrait ou un composé d'un pathogène ou un fragment ou dérivé de celui-ci.

8. Vaccin selon l'une quelconque des revendications 1 à 7, dans lequel le composé exerçant une activité cytokine est IL-4, IL-2, TGF, IL-6, TNF, IL-1α et IL-1β, un interféron de type I, de préférence IFN-α ou IFN-β, IL-12, IFN-γ, TNF-β, IL-5 ou IL-10.

9. Vaccin selon l'une quelconque des revendications 1 à 8, dans lequel le composé présentant une activité anticytokine est un anticorps anticytokine ou le fragment actif correspondant, un dérivé ou un analogue de celui-ci.

10. Vaccin selon l'une quelconque des revendications 1 à 9, dans lequel l'antigène est dérivé d'un pathogène.

11. Vaccin selon la revendication 10 dans lequel ledit pathogène est choisi parmi des bactéries extracellulaires, comprenant les cocci formant du pus, tels que *Staphylococcus* et *Streptococcus,* des bactéries gram négatif, telles que les espèces Meningococcus et Gonococcus, les espèces de *Neisseria,* des bactéries gram négatif, comprenant des organismes entériques tels que *E. coli, Salmonella, Shigella, Pseudomonas, Diphteria, Bordetella, Pertussis,* et des bactéries gram positif (par exemple, *Bacillus pestis, BCG*), en particulier des anaérobies tels que les espèces *Clostridium*, les bactéries et les virus, qui survivent et se répliquent à l'intérieur des cellules hôtes, comprenant les mycobactéries (par exemple, *M. tuberculosis*) et *Listeria monocytogenes,* des rétrovirus et adénovirus, comprenant le virus de l'hépatite (humain), le virus d'immunodéficience, les virus de l'herpès, les virus de la petite vérole (varicelle), la grippe, la rougeole, les oreillons et la polio, le cytomégalovirus, le rhinovirus, etc., et les champignons proliférant à l'intérieur de cellules hôtes, les parasites comprenant des parasites animaux, tels que les protozoaires et les helminthes, et les ectoparasites, tels que les tiques et les mites, ou les espèces de *Brucella,* comprenant l'agent causal du choléra, les espèces de Haemophilius, ainsi que des pathogènes déclenchant les maladies paratyphoïde, peste, rage, tétanos et rubéole et les pathogènes provoquant différentes néoplasies, des maladies auto-immunes ou autres associées à d'autres états pathologiques du corps animal ou humain qui ne résultent pas nécessairement d'infections pathogènes.

12. Vaccin selon l'une quelconque des revendications 1 à 11, dans lequel l'allergène est d'origine xénogène ou endogène, dérivé d'un microorganisme, un animal ou une plante, ou appartenant au groupe des substances synthétiques et/ou irritantes inorganiques, ou à des parties ou composants du corps humain qui ont été incorrectement traités par ou exposés au système immunitaire corporel.

13. Vaccin selon l'une quelconque des revendications 1 à 12, dans lequel la concentration de chaque composé présentant une activité cytokine utilisée est choisie de façon à être jusqu'à 1000 fois plus forte que la concentration optimale déterminée dans les essais correspondants avec la dose d'antigène et l'immunoadjuvant choisi, conduits en injectant la formule ou en conduisant les essais *in vitro,* et préférablement jusqu'à 100 fois, plus préférablement jusqu'à 50 fois, et encore plus préférablement jusqu'à 20 fois plus élevée.

14. Vaccin selon l'une quelconque des revendications 7 à 13, dans lequel l'extrait ou composé de pathogène est un lipopolysaccharide, le facteur médullaire dimycolate de tréhalose), un dipeptide de muramyle, ou un autre (poly) saccharide ou (poly)peptide identique à ou ressemblant à une partie immunologiquement active d'une membrane d'un pathogène ; un extrait d'un pathogène, comprenant des exo- et endotoxines bactériennes, de préférence la toxine du choléra et la toxine thermolabile de *E. coli,* un dérivé de chaîne A, un composant ayant une activité ADP-ribosylante, un peptidoglycan, une toxine clostridienne, ou un dérivé de protéine purifié de *M. tuberculosis,* LT-R192G, la protéine fixant la fibronectine I de *Streptococcus pyrogenes,* ou une protéine de membrane externe de *Neisseria meningitidis* groupe B (GBOMP).

15. Vaccin selon la revendication 14 dans lequel ledit lipopolysaccharide est le lipide A ou un dérivé et une modification de celui-ci, tel qu'un monophosphoryllipide A, ou son analogue, tel qu'un dérivé lipidique du saccharose.

16. Vaccin selon l'une quelconque des revendications 7 à 13, dans lequel la concentration du composé pathogène dérivé d'un pathogène est 10 fois plus faible à 1000 fois plus forte que celle utilisée avec les formulations injectées correspondantes utilisant un antigène similaire, la concentration d'immunoadjuvant administrée par voie épicutanée différant plus souvent de la concentration d'immunoadjuvant injectée d'un facteur compris entre 0,5 et 100, ou plus préférablement, d'un facteur compris entre 1 et 50, et le plus préférablement entre 2 et 25.

17. Vaccin selon l'une quelconque des revendications 7 à 16, dans lequel ledit irritant à bas poids moléculaire est choisi parmi les classes d'ions de métal, acides, bases, liquides irritants, alcools (gras), amines (grasses), éthers (gras), sulfonates (gras), phosphates, etc. allergènes, ou d'autres solvants ou amphiphiles adaptés, ou dans le groupe des molécules de type tensioactif, présentant souvent la capacité d'améliorer la perméation de la peau, ainsi que des dérivés ou combinaisons de ceux-ci.

18. Vaccin selon l'une quelconque des revendications 7 à 17, dans lequel la concentration d'un irritant à bas poids moléculaire est choisi pour être d'au moins un facteur 2, plus préférablement d'un facteur 5, et encore plus préférablement d'un facteur 10 ou plus, inférieure à la concentration qui, dans des essais indépendants sur un sujet identique ou comparable est jugée inacceptable en raison de l'irritation locale, comme déterminé par les méthodes et standards couramment utilisés pour tester un tel irritant.

19. Vaccin selon l'une quelconque des revendications 7 à 16, dans lequel l'allergène appartient à la classe des allergènes par inhalation, comprenant sans y être limités différentes pollens, spores, fragments de poil, peau, plumes d'animal, textiles naturels et synthétiques, blé, poussières (domestiques), comprenant les mites ; de plus, des allergènes alimentaires et pharmaceutiques ; des allergènes de contact ; des allergènes par injection, invasion ou dépôt, tels que différents vers (résidant dans le tube digestif), echinococcus, trichines, etc., un fragment de matériau d'implantation.

20. Vaccin selon l'une quelconque des revendications 1 à 19, dans lequel la dose appliquée d'un antigène diffère d'un facteur de 0,1 à 100 de la dose qui aurait dû être injectée dans le processus d'immunisation, mais plus souvent est dans l'intervalle compris entre 0,5 et 50, plus préférablement entre 1 et 20 et idéalement, est inférieur à 10 fois plus que celle qui est utilisée avec une injection.

21. Vaccin selon l'une quelconque des revendications 1 à 20, dans lequel la dose pénétrante appliquée est comprise entre 0,1 $mg.cm^{-2}$ et 15 $mg.cm^{-2}$, plus souvent dans l'intervalle de 0,5 $mg.cm^{-2}$ et 10 $mg.cm^{-2}$, et de préférence entre 1 $mg.cm^{-2}$ et 5 $mg.cm^{-2}$.

22. Vaccin selon l'une quelconque des revendications 1 à 21, dans lequel ledit antigène est un antigène pur ou purifié.

23. Kit comprenant, sous une forme flaconnée ou un autre conditionnement, au moins une dose du vaccin selon l'une quelconque des revendications 1 à 22.

24. Kit selon la revendication 23 comprenant également au moins une dose injectable de l'antigène spécifié dans la revendication 11 ou de l'allergène spécifié dans la revendication 12.

25. Utilisation d'un vaccin selon l'une quelconque des revendications 1 à 22 pour la préparation d'une composition pharmaceutique pour générer une réponse immunoprotectrice chez un mammifère.

26. Utilisation selon la revendication 25 dans laquelle différents domaines de traitement sont sélectionnés pour contrôler la dose d'immunogène appliquée et le résultat de la vaccination thérapeutique.

27. Utilisation selon la revendication 25 ou 26, dans laquelle une suspension de pénétrants sans antigène est chargée avec l'antigène pour être associée à celui-ci pendant le jour précédant l'administration, de préférence 360 min, plus préférablement 60 min et encore plus préférablement 30 min avant l'administration de la formulation résultante sur la peau.

28. Utilisation selon l'une quelconque des revendications 25 à 27, dans laquelle le vaccin selon l'une quelconque des revendications 1 à 22 doit être appliqué sur la peau après prétraitement de l'organe par une manipulation immunoadjuvante, ladite manipulation comprenant, par exemple, abrasion, pression, chauffage, exposition à un champ électrique ou mécanique, des ultrasons par exemple, etc. de la peau ou l'injection d'une formule non immunogène dans la peau, à condition qu'un tel traitement libère des composés immunoadjuvants depuis la peau ou d'autres tissus immunoactifs périphériques ou diminue autrement la concentration / durée d'action d'antagonistes de la vaccination souhaitée.

29. Utilisation selon l'une quelconque des revendications 25 à 28, dans laquelle l'immunogène doit être appliqué dans un patch non occlusif.

30. Utilisation selon l'une quelconque des revendications 25 à 29, **caractérisée en ce qu'**au moins une dose de vaccin doit être administrée.

31. Utilisation selon la revendication 30 dans laquelle ledit vaccin doit être administré comme vaccination de rappel.

32. Utilisation selon la revendication 31, dans lequel l'immunisation primaire est effectuée de manière invasive, généralement en utilisant une injection sous-cutanée ou tout autre méthode de perforation/destruction de la barrière cutanée adaptée, et dans laquelle la au moins une immunisation de rappel ultérieure doit être effectuée de manière non invasive.

33. Utilisation selon l'une quelconque des revendications 25 à 32, dans laquelle le vaccin doit être appliqué entre 2 et 10, de préférence entre 2 et 7, encore plus préférablement jusqu'à 5 et plus préférablement jusqu'à 3 fois, quand un antigène non allergène est utilisé, ou un nombre de fois tel, dans le cas d'allergènes, requis pour obtenir l'immunotolérance souhaitée, déterminé suivant une méthode d'évaluation adaptée, ou sinon pour estimer l'action comme ayant échoué.

34. Utilisation selon la revendication 33, dans laquelle l'intervalle de temps entre les vaccinations successives est choisi pour être entre 2 semaines et 5 ans, souvent entre 1 mois et jusqu'à 3 ans, plus fréquemment entre 2 mois et 1,5 ans.

35. Utilisation selon l'une quelconque des revendications 25 à 34, dans laquelle le flux de pénétrants qui transporte un immunogène par l'intermédiaire des différents pores dans une barrière bien définie doit être déterminé en fonction d'une force d'entraînement ou d'une pression adaptée exercée à travers la barrière et les données sont ensuite commodément décrites par une courbe caractéristique qui est ensuite utilisée pour optimiser plus avant la formule ou application.

36. Utilisation du porteur transdermique, du composé qui libère spécifiquement ou induit spécifiquement l'activité cytokine ou anticytokine ou exerce une telle activité lui-même, de l'antigène ou allergène, et facultativement d'un extrait ou d'un composé d'un microorganisme, ou un fragment ou dérivé de celui-ci, et/ou un irritant chimique à bas poids moléculaire comme défini dans l'une quelconque des revendications précédentes pour la préparation d'un vaccin pour induire une réponse immunitaire protectrice ou tolérogène.

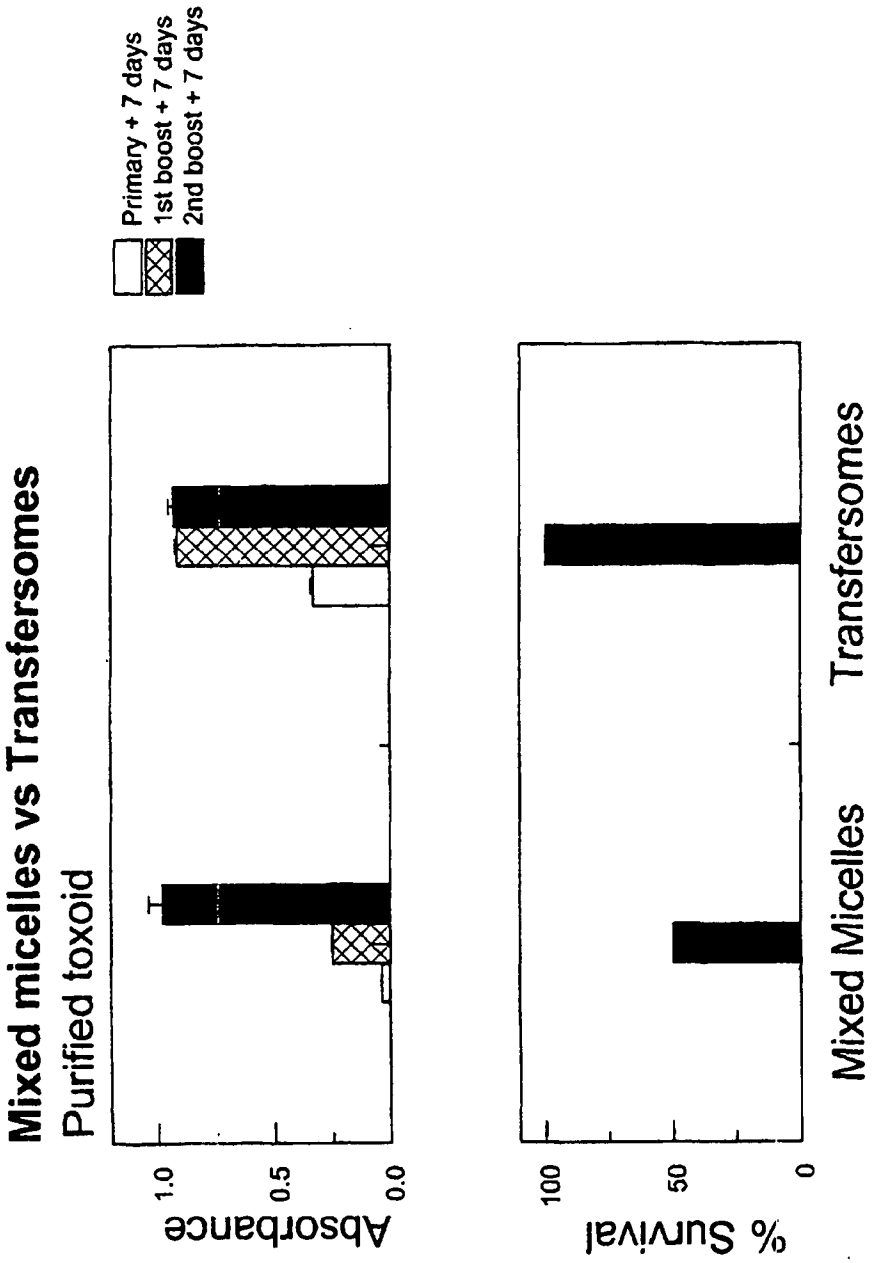

Mixed micelles vs Transfersomes
Purified toxoid
Primary + 7 days
1st boost + 7 days
2nd boost + 7 days
Absorbance
1.0
0.5
0.0
% Survival
100
50
0
Mixed Micelles
Transfersomes

Figure 1

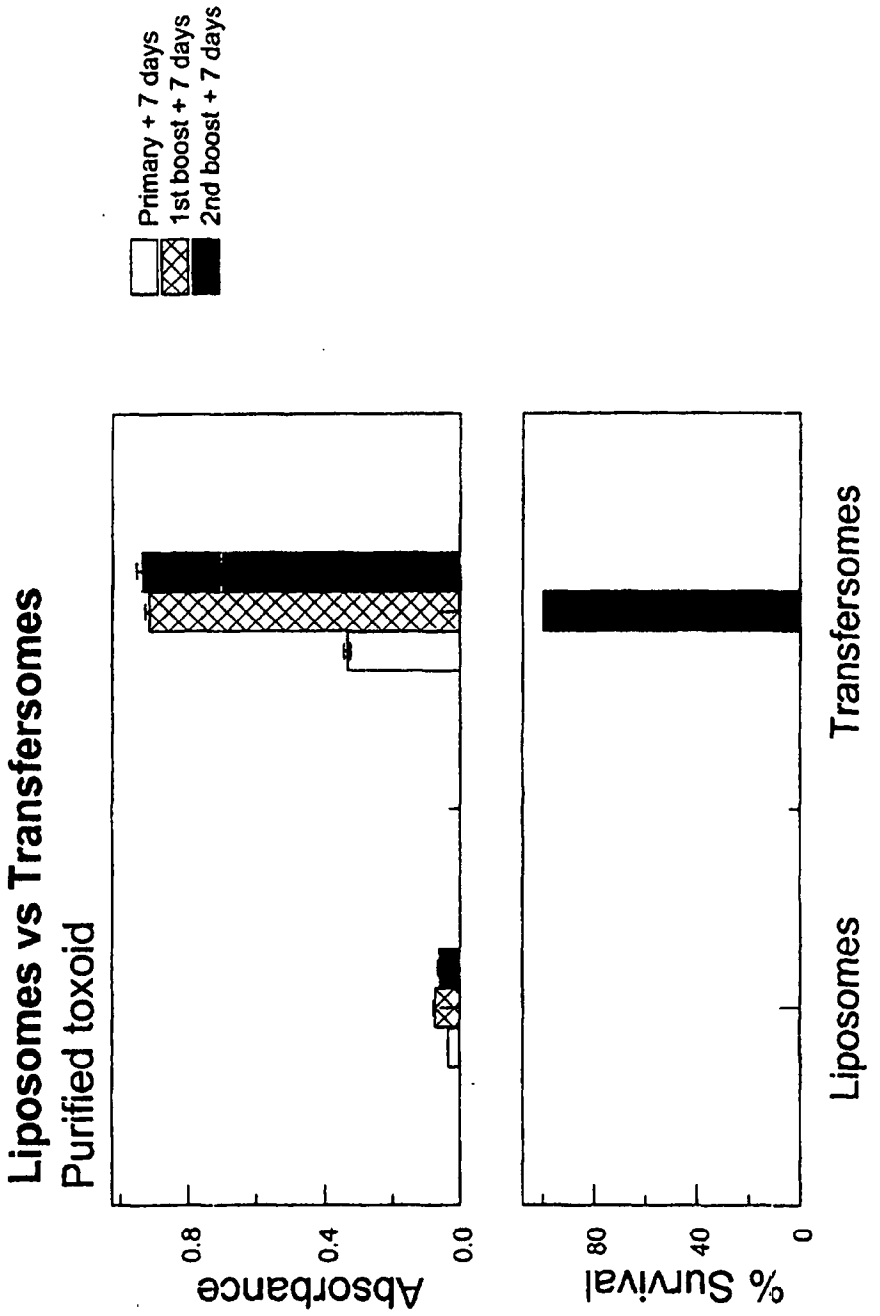

Liposomes vs Transfersomes
Purified toxoid
Primary + 7 days
1st boost + 7 days
2nd boost + 7 days
Absorbance
0.0
0.4
0.8
% Survival
0
40
80
Liposomes
Transfersomes

Figure 2

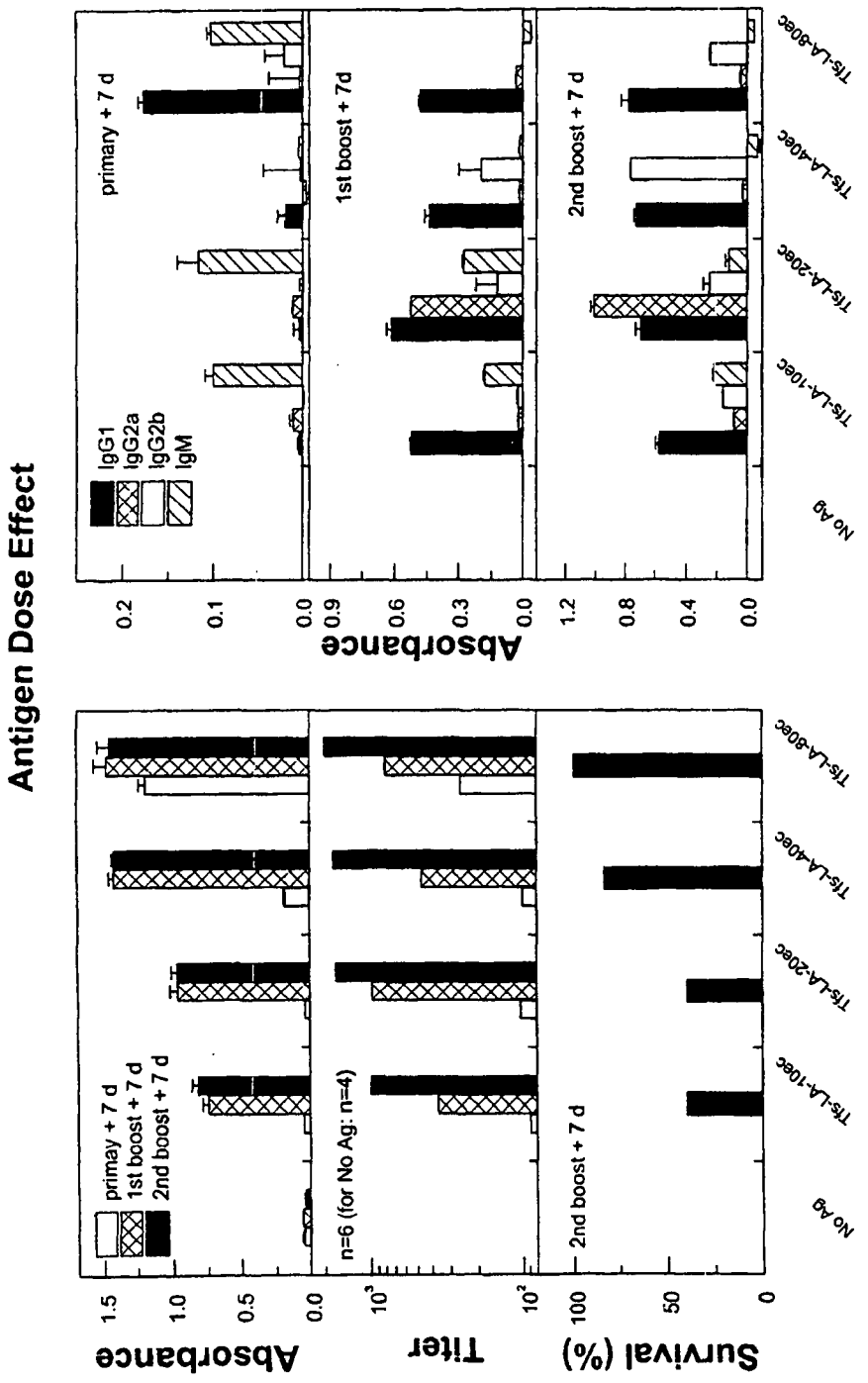

Antigen Dose Effect
primay + 7 d
1st boost + 7 d
2nd boost + 7 d
IgG1
IgG2a
IgG2b
IgM
Absorbance
Titer
Survival (%)
n=6 (for No Ag: n=4)
2nd boost + 7 d
primary + 7 d
1st boost + 7 d
No Ag
Tfs-LA-10ec
Tfs-LA-20ec
Tfs-LA-40ec
Tfs-LA-80ec
antigen doses: 10, 20, 40 and 80 µg
LA: Monophosphoryl Lipid A
Tfs: Transfersomes (SPC:NaChol 3.75:1)

Figure 3

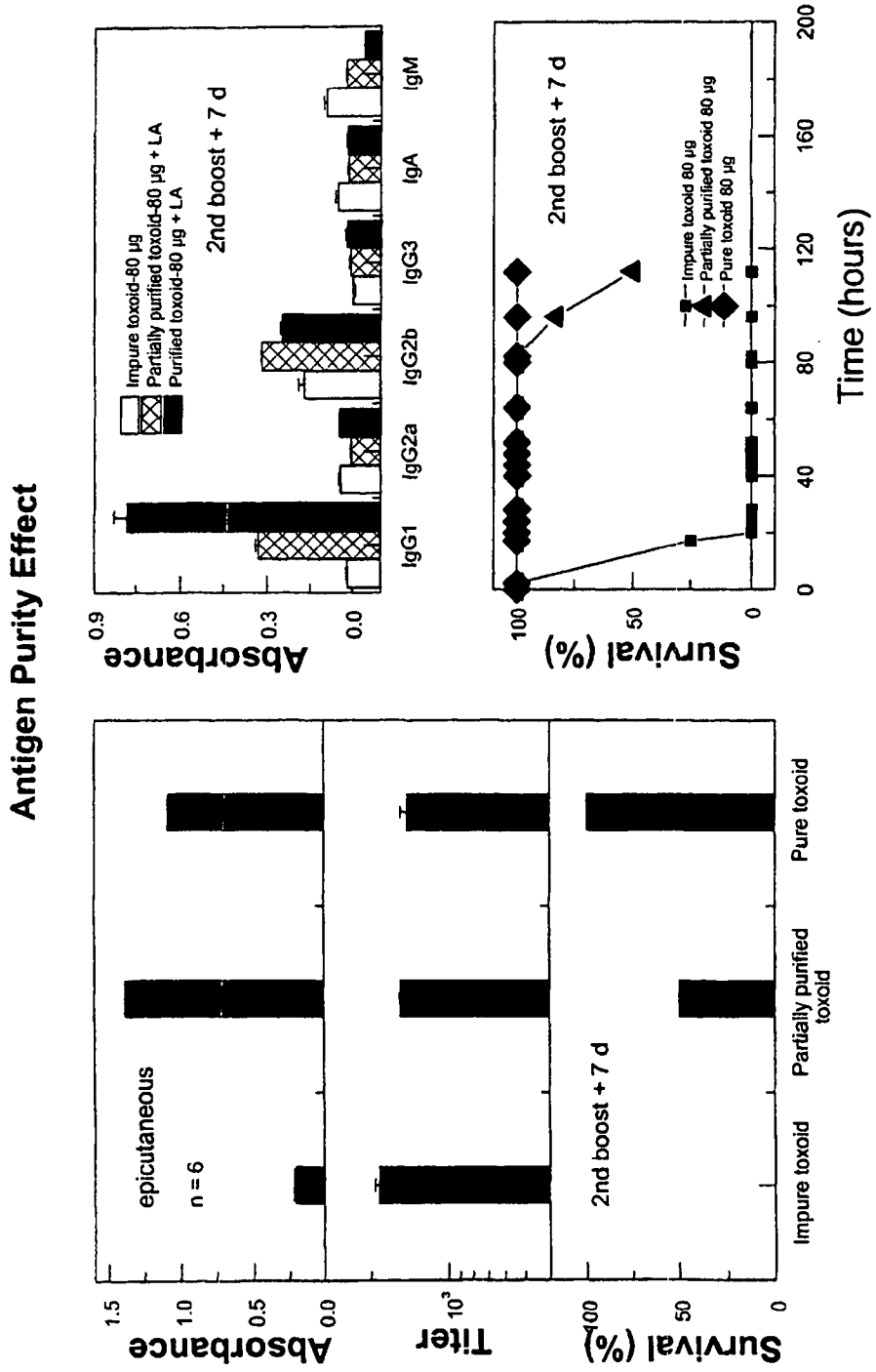

Antigen Purity Effect
epicutaneous
n = 6
Absorbance
Titer
Survival (%)
Impure toxoid
Partially purified toxoid
Pure toxoid
2nd boost + 7 d
Impure toxoid-80 µg
Partially purified toxoid-80 µg + LA
Purified toxoid-80 µg + LA
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
Impure toxoid 80 µg
Partially purified toxoid 80 µg
Pure toxoid 80 µg
Time (hours)
Immunized with Tfs (SPC:NaCh 3.75:1)
containing 80 µg toxoid
LA: Monophosphoryl lipid A

Figure 4

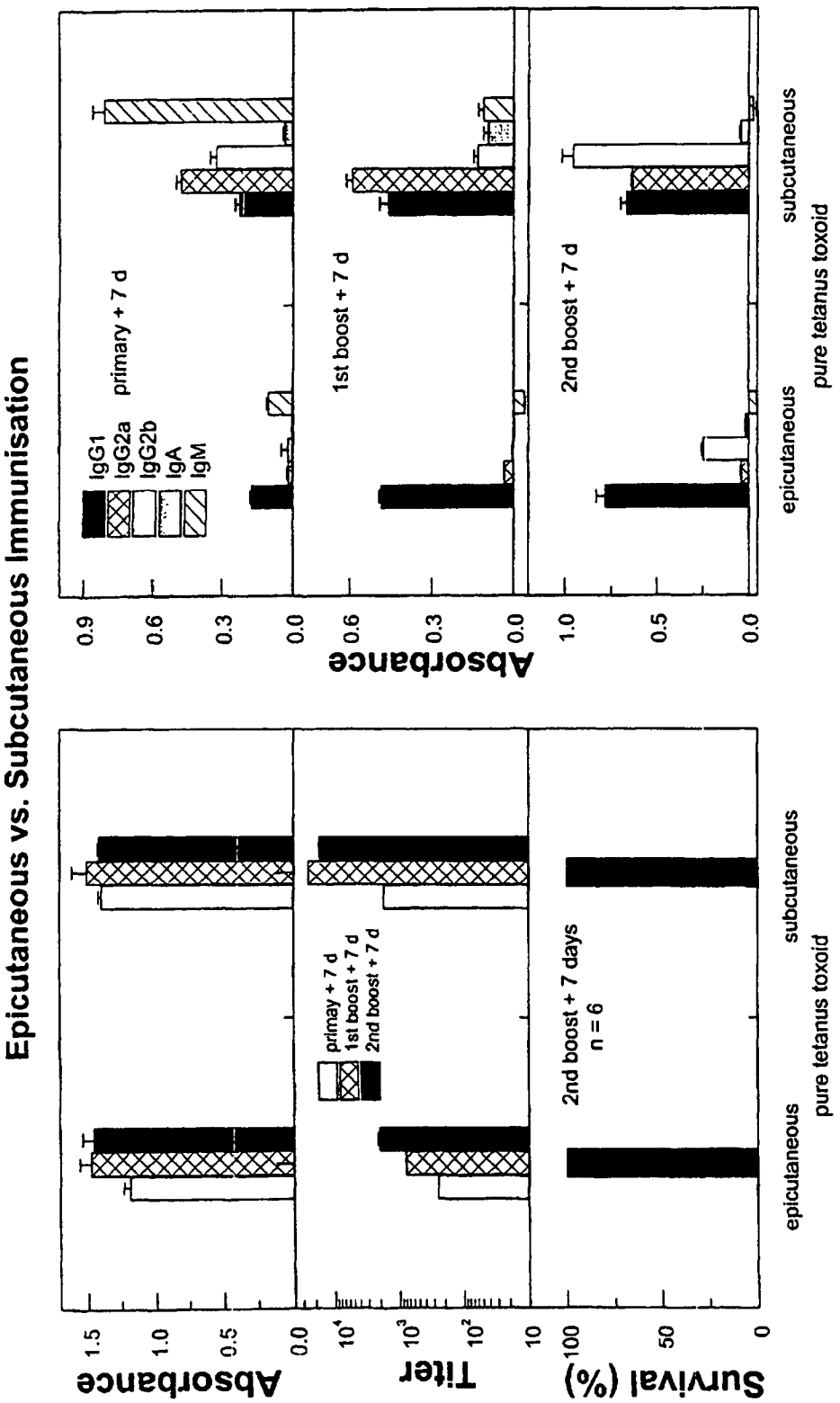

Epicutaneous vs. Subcutaneous Immunisation
Absorbance
Titer
Survival (%)
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
IgG1
IgG2a
IgG2b
IgA
IgM
2nd boost + 7 days
n = 6
epicutaneous
subcutaneous
pure tetanus toxoid

Figure 5

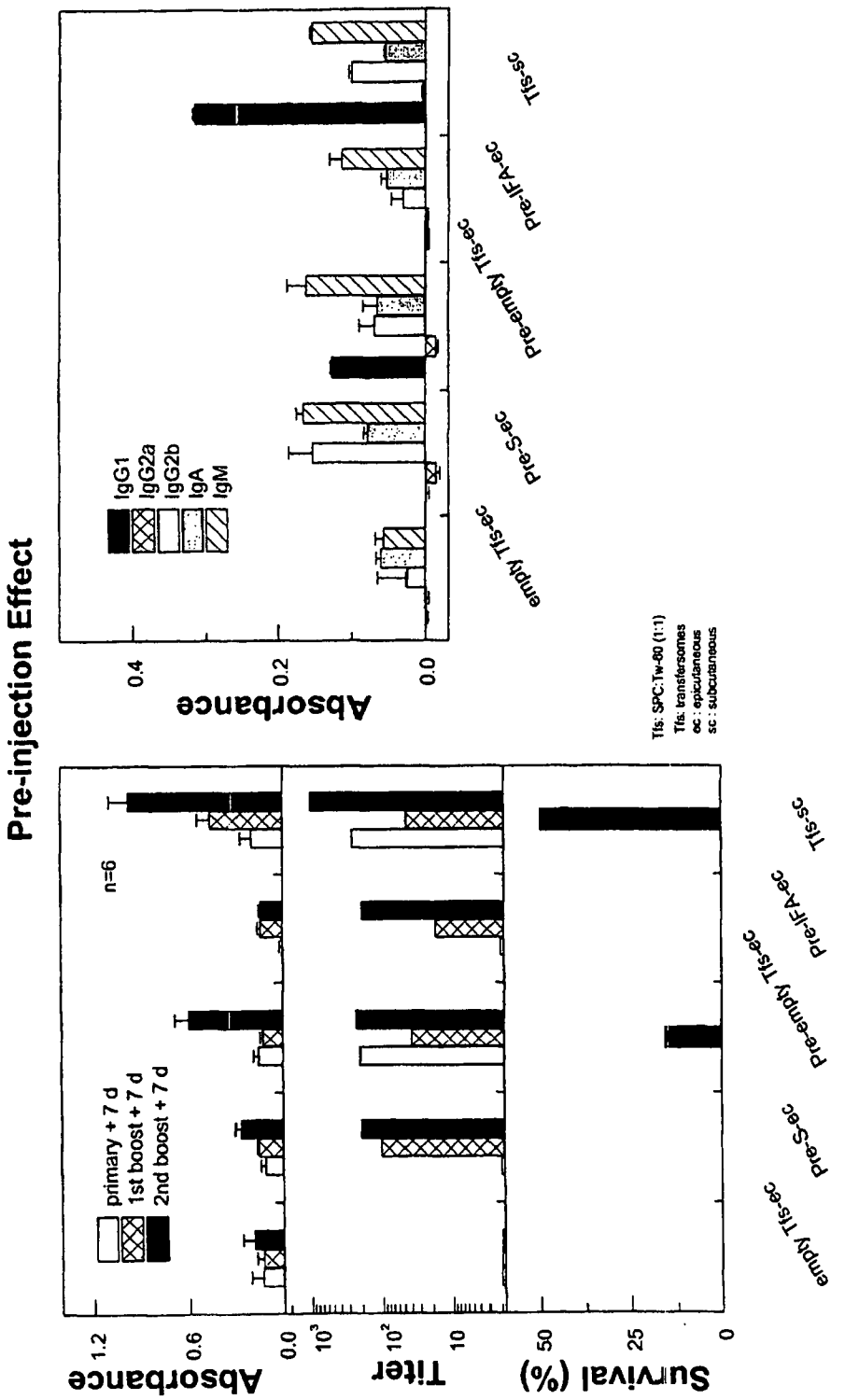


Figure 6

Mice in the preinjection groups were injected 21 hours before the application at 40 µg antigen. 0.1 ml, each of saline, 10% SPC:NaCl (4:5.1) empty transfersomes and incomplete Freund's adjuvant were used for pre-injection. All the mice in this experiment were challenged with 50 times LD_{50} dose of toxin 7 days after the second boost

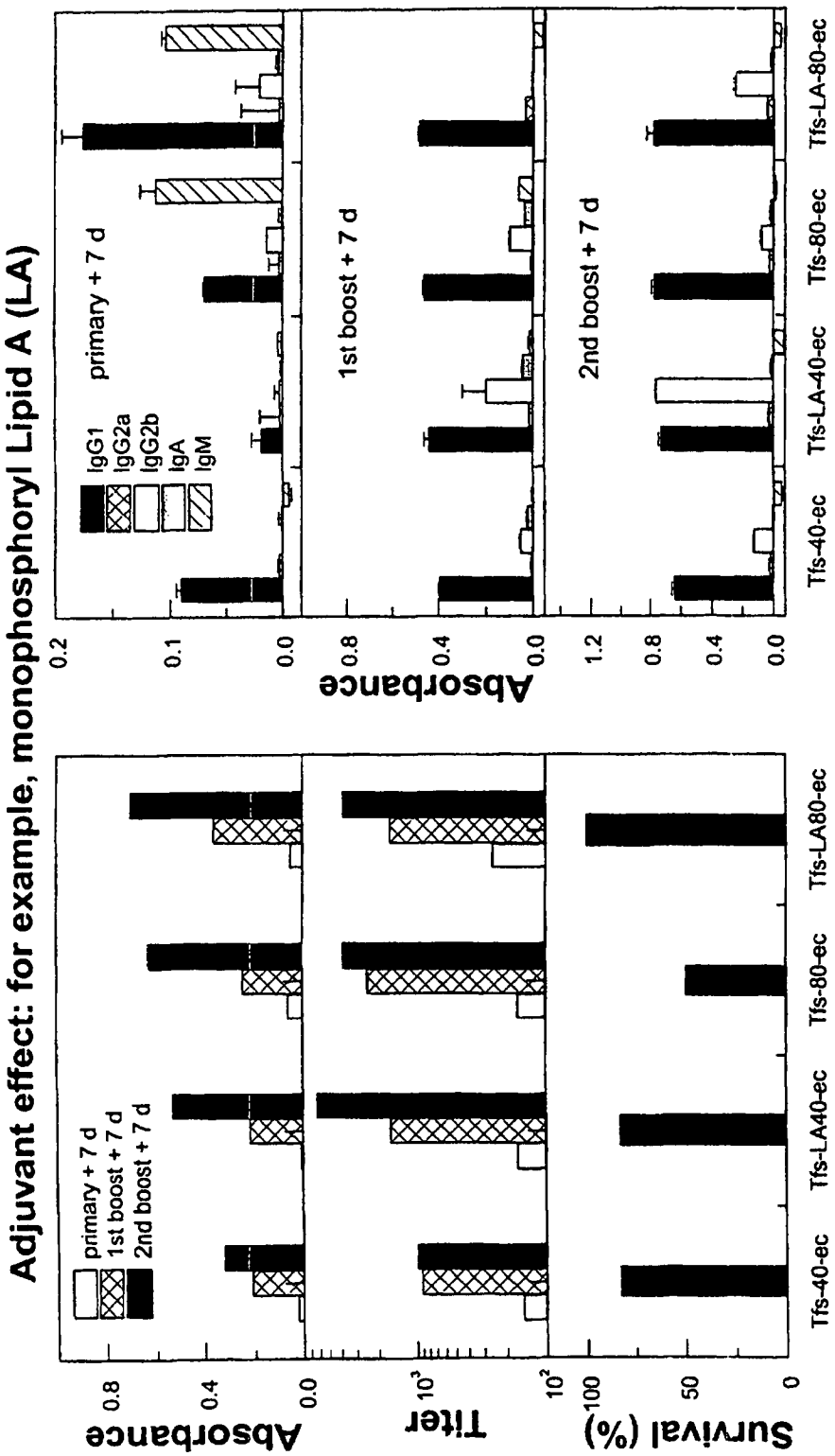

Adjuvant effect: for example, monophosphoryl Lipid A (LA)
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Absorbance
Titer
Survival (%)
Tfs-40-ec
Tfs-LA40-ec
Tfs-80-ec
Tfs-LA80-ec
* partially paralyzed but alive
IgG1
IgG2a
IgG2b
IgA
IgM
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Absorbance
Tfs-40-ec
Tfs-LA-40-ec
Tfs-80-ec
Tfs-LA-80-ec

Figure 7

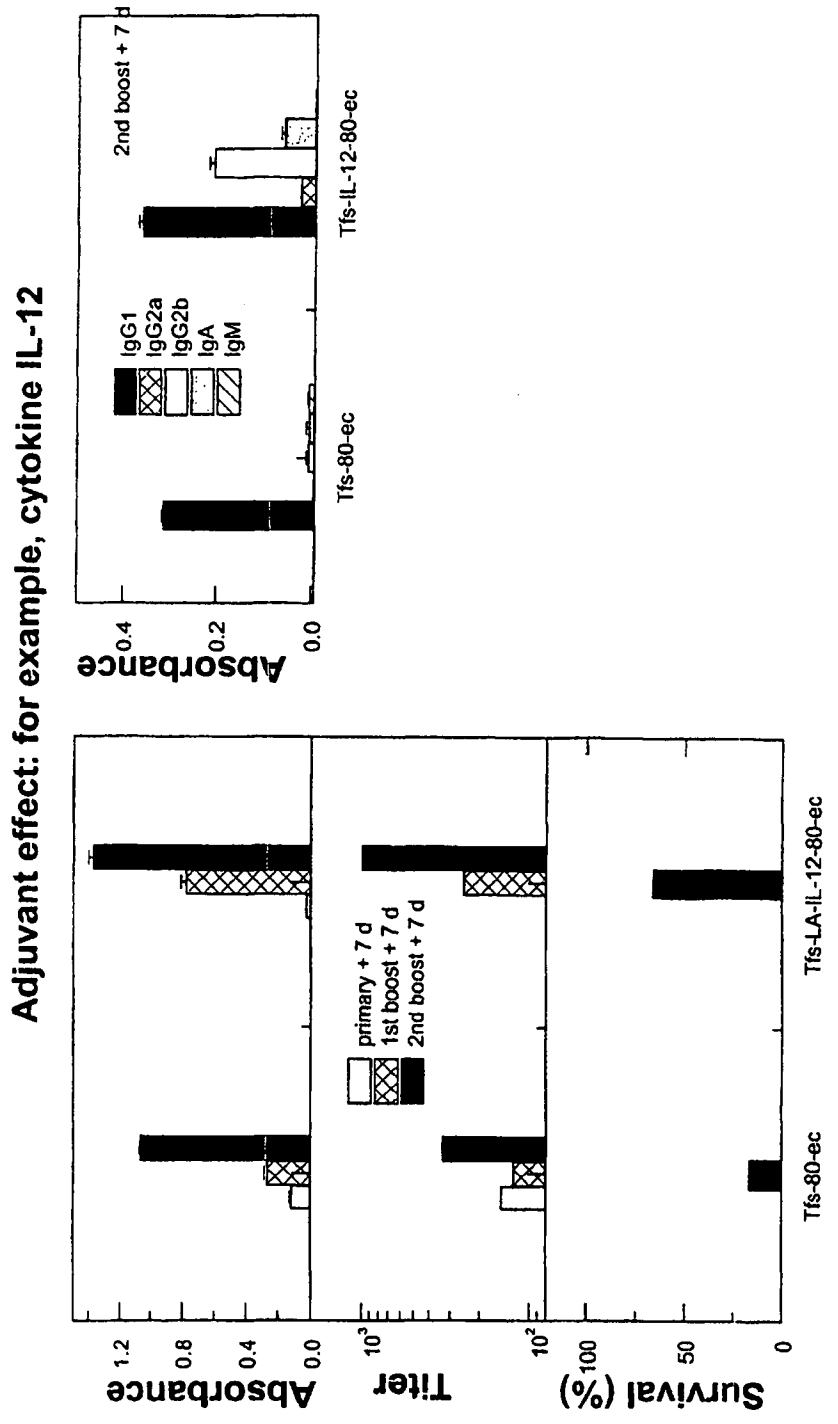

Adjuvant effect: for example, cytokine IL-12
Absorbance
Titer
Survival (%)
primary + 7 d
1st boost + 7 d
2nd boost + 7 d
Tfs-80-ec
Tfs-LA-IL-12-80-ec
IgG1
IgG2a
IgG2b
IgA
IgM
2nd boost + 7 d
Tfs-80-ec
Tfs-IL-12-80-ec
Tfs: SPC:NaCh ec: epicutaneus

Figure 8

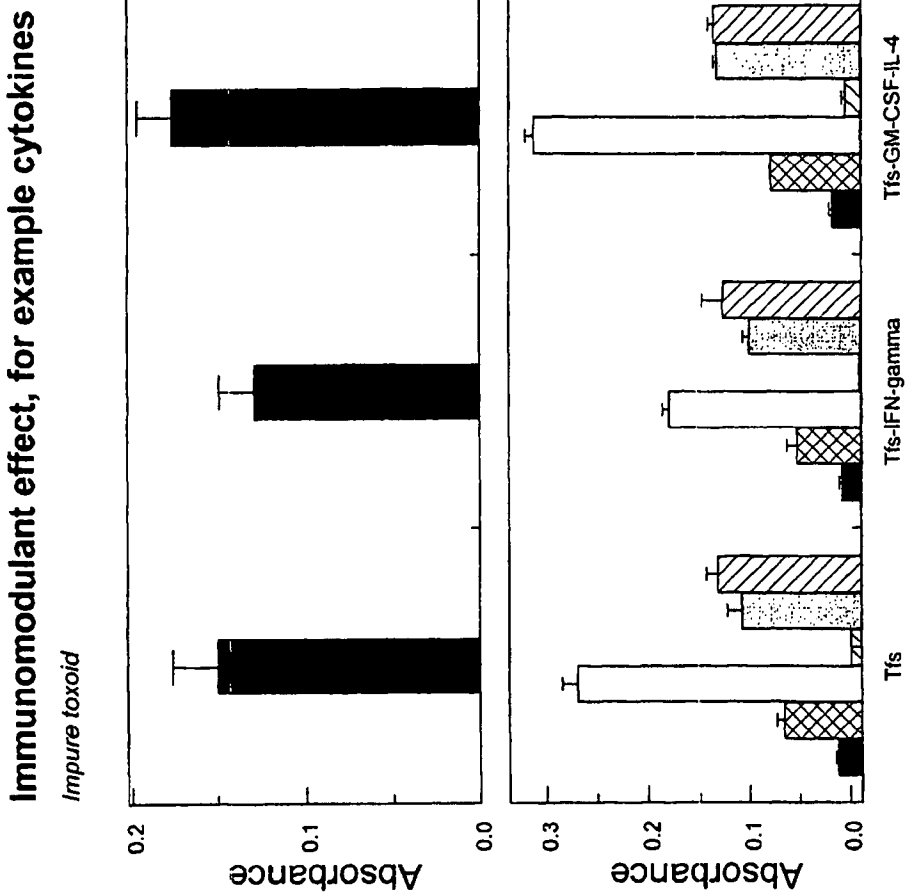
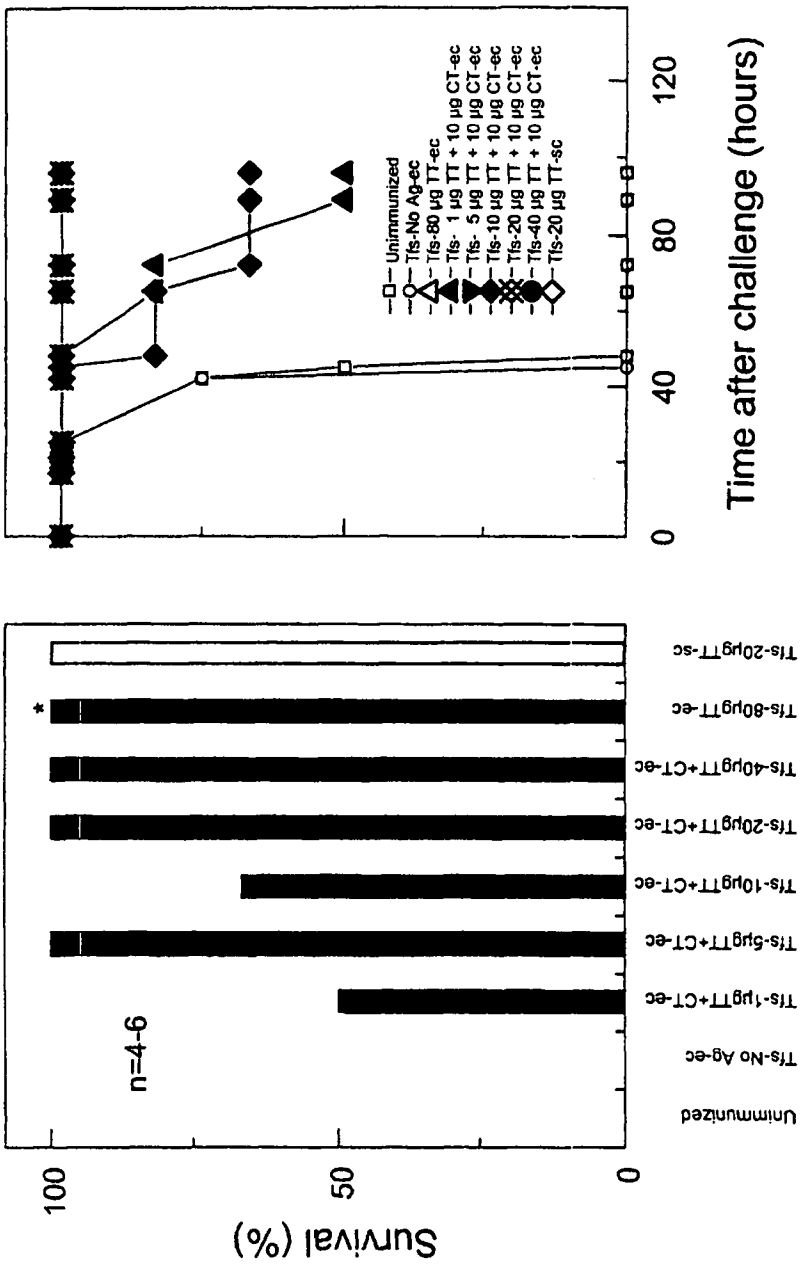


Figure 9

Serum was collected for the assay on 7th day after 2nd boost
No protection was observed in any of the groups.

Immunoadjuvant effect: for example, cholera toxin (CT)
added to tetanus toxoid (TT, pure) in Transfersomes (Tfs) on the skin



Tfs: SPC:Sodium Cholate(3.75:1)
CT dose. 10 µg per mouse wherever used
n=4-6
* 1 mouse partially paralyzed out of 4

Figure 10